CHARACTERISATION OF WINE YEASTS FOR VARIETAL RED WINE PRODUCTION BY USING CHEMICAL, SENSORY AND METABOLOMIC TOOLS

by

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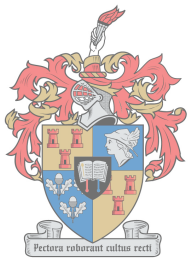

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Modern day wine making includes direct inoculation of active dried yeast (ADY), primarily *Saccharomyces cerevisiae*, into relatively 'neutral' flavoured grape must. Subsequently, wine yeast strains influence wine quality through *de novo* synthesis or by converting odourless aroma precursors present in red grape must into aroma active compounds, which contribute to the varietal aromas and flavours ranging from 'strawberry', 'raspberry', 'blackcurrant', 'plum', 'caramel', 'herbaceous and/or vegetative', to 'spicy', and even 'peppery'. Furthermore, yeast proteins produced and secreted during alcoholic fermentation were shown to have oenological importance, since they are critical during the release of some aroma compounds *e.g.* volatile thiols. Thus, it is important to select yeast starter cultures with the ability to enhance and complement varietal aromas and flavours. Therefore, this master's study was undertaken with the aim of investigating the influence of a naturally isolated wine yeast strain *i.e.* ARC Nvbij 6 (*S. cerevisiae*) on typical red wine quality by utilising chemical, sensory, proteomic and metabolomics characterisation tools. Shiraz, Merlot and Cabernet Sauvignon winemaking trials were initiated during the 2016 and 2017 vintages with the inclusion of two commercial reference strains *i.e* WE372 (Anchor Oenologies, South Africa) and MERIT (Chr. Hansen, Denmark). The yeast strain ARC Nvbij 6 was shown to consistently produce Shiraz, Merlot, and Cabernet Sauvignon during the 2016 and 2017 vintages, equal and in some instances better than both commercial references. It is noteworthy that all wines produced with ARC Nvbij 6 also had a negative association with undesirable volatile acidity (VA) and acetic acid, which are known to impart unpleasant off-odours, thereby masking the sought-after varietal aromas and flavours. Furthermore, descriptive sensory evaluations showed that the ARC Nvbij 6 strain, for the most part, produced Shiraz, Merlot, and Cabernet Sauvignon wines with sought-after aromas and flavours. Gas chromatography (GC) also showed the ARC Nvbij 6 strain to be a better '3-mercaptohexan-1-ol (3MH) to 3-mercaptohexyl acetate (3MHA) converter', as both commercial references also failed to convert 3MH to 3MHA during one vintage in two cultivars. In terms of aroma compounds *i.e.* esters (associated with fruity nuances), both commercial references mostly produced Shiraz, Merlot and Cabernet

Sauvignon wines with higher ester concentrations than the ARC Nvbij 6 strain. Nonetheless, ARC Nvbij 6 consistently produced less of the undesirable compounds that are associated with wine off-odours, which can influence the wine sensory quality negatively. Furthermore, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that all yeast strains differentially expressed proteins within given molecular weights. It can be envisaged that peptide mass fingerprinting (PMF) in conjunction with matrix-assisted laser desorption ionization with time of flight mass spectrometry (MALDI -TOF MS) will be deployed to characterise specific yeast-derived proteins that were regulated and draw conclusions with regard to how they are associated with aroma compounds. Thus, proteomic tools may be used to select promising wine yeast strains with sought-after traits in terms of wine quality. The use of multiple omics approaches is also encouraged, as proteome does affect metabolome, which in turn determine wine chemical and sensory quality. Overall, the ARC Nvbij 6 strain proved that it has a commercial role to play in the production of varietal red wines, especially Shiraz, based on chemical and sensory attributes of all red wines included in this study.

OPSOMMING

Moderne wynmaak behels direkte inenting van ‘neutral’ gegeurde druiwemos met aftief gedroogde gis (AGG), hoofsaaklik *Saccharomyces cerevisiae*. Gevolglik, affekteer bovermelde gisras wyn kwaliteit deur *de novo* sintese of omskakeling van geurlose aroma verbindings afkomstig vanaf druiwe na vlugtige aromatiese verbindings wat bydra tot kultivar aroma and geure, onder andere, ‘aarbei’, ‘framboos’, ‘swartbessie’, ‘pruim’, ‘karamel’, ‘kruidagtig/vegetatief’, ‘speserye’, en selfs ‘pepper’. Gis-geproduseerde en uitgeskeide proteïene tydens alkoholiese fermentasie is van oenologiese belang, siende dat dit ‘n rol speel tydens vrystelling van sommige aroma verbindings byvoorbeeld vlugtige tiole. Die seleksie van gis suursel kulture met die vermoë om kultivar aromas en geure uit te lig is dus belangrik. Op grond hiervan is ‘n meesters studie onderneem met die doel om die effek van ‘n natuurlik geïsoleerde wyngis naamlik ARC Nvbij 6 (*S. cerevisiae)* op tipiese rooiwyn kwaliteit te ondersoek met behulp van chemiese, sensoriese, proteïen en metaboliet evaluasies. Gevolglik is Shiraz, Merlot en Cabernet Sauvignon wynmaak proewe tydens 2016 en 2017 oesjare geinisieër, met die insluiting van twee kommersiële verwyssings gisrasse naamlik WE372 (Anchor Oenologies, South Africa) en MERIT (Chr. Hansen, Denmark). Die gisras ARC Nvbij 6 het konsekwent Shiraz, Merlot en Cabernet Sauvignon wyne gelyk en soms beter in kwaliteit as beide verwyssings giste geproduseer gedurende beide oesjare (2016 en 2017). Dit is opmerklik dat die ARC Nvbij 6 gisras rooiwyne produseer het wat `n negatiewe assosiasie met ongewenste vlugtige suur (VS) sowel as asynsuur getoon het. Beide verbindings dra by tot onsmaaklike afgeure, wat op hul beurt gesogte kultivar aromas en geure oordonder. Beskrywende sensoriese evaluerings het ook getoon dat ARC Nvbij 6 Shiraz, Merlot en Cabernet Sauvignon wyne produseer het met gesogte kultivar aromas en geure. Verdermeer het gas chromatografiese (GC) analise ook gewys dat die gis ‘n doeltreffender ‘3-merkaptoheksanol (3MH) na 3-merkaptohexyl asetaat (3MHA)’ omskakkelaar is in vergelyking met beide kommersiële verwyssings giste. Laasgenoemde giste het wel Shiraz, Merlot en Cabernet Sauvignon wyne produseer met hoër ester (word geassosieer met vrugtige geure) vlakke as wat ARC Nvbij 6 geproduseer het. Die gisras ARC Nvbij 6 het nogtans konsekwent

aansienlik minder ongewenste verbindings wat rooiwyn sensoriese kwaliteit negatief kan beïnvloed geproduseer. Natrium dodecyl sulfaat poli-akrielamied gel elektroforese (SDS-PAGE) het ook getoon dat alle giste proteïne met gegewe molekulere gewigte differensieël uitgedruk het. Daar word ook onderneem om spesifieke gereguleerde gis proteïene te karakteriseer met behulp van peptied massa vingermerking (PMF) en matriks-geassesteerde desorpsie ionisasie met tyd van vlug massa spektrometrie (MALDI-TOF). Daarvolgens kan gevolgtrekkings gemaak word of bovermelde proteïne enigsins ‘n assosiasie het met aroma verbindings. Dit wil blyk asof proteïn analitiese metodes ‘n rol kan speel tydens die seleksie van belowende wyngisrasse met gesogte kenmerke in terme van wynkwaliteit. Die gebruik van veelvuldige ‘omics’ benaderings word ook aanbeveel, siende dat proteïen uitdrukking metaboliet produksie en vrystelling affekteer, wat op hul beurt wyn chemiese en sensoriese kwalieit bepaal. Oor die algemeen wys die studie dat ARC Nvbij 6 ‘n kommersiële rol het om te speel vir die produksie van eiesoortige rooiwyn, veral Shiraz op grond van chemiese en sensoriese eienskappe van alle rooiwyn kultivars wat in hierdie studie ingelsuit is.

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In loving memory of my sister Nicolene Leonard RIP

PREFACE

This thesis is presented as a compilation of four chapters.

Chapter 1: Introduction and project aims

Chapter 2: Literature review

Influence of *Saccharomyces cerevisiae* on red wine aroma and flavour

Chapter 3: Research results

Characterisation and evaluation of wine yeast used for the production of typical varietal red wines

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Chapter 1

Introduction and Aims

CHAPTER 1: INTRODUCTION AND AIMS

1.1 BACKGROUND

Wine production plays an integral part of the agricultural sector in South Africa since wine exports contribute billions to the Gross Domestic Product (GDP). In 2013, the wine industry contributed R36 145 billion to the annual GDP of South Africa (SAWIS, 2015) and it is clear that the wine industry greatly contributes to the economy of this country. Wine, however, cannot exist without yeast and these microbes are in fact of cardinal importance for the production of varietal wines. Therefore, it is important to select yeasts that adhere to certain criteria and that are able to complement grape quality and the specific varietal characters (flavours and aromas) associated with the respective grape cultivars.

Modern day wine making includes direct inoculation of active dried yeast, primarily *Saccharomyces cerevisiae*, into grape must (Suárez-Lepe and Morata, 2012). This method produces fast, predictable and reproducible fermentations in comparison to spontaneous fermentation (Pretorius, 2000). Furthermore, wine yeast strains influence the quality of the wine by producing aroma active compounds, which contribute to the varietal aroma of the wine (Loscos et al., 2007; Hernandez-Orte et al., 2008). This varietal aroma originates from the grape cultivar, which gives the wine its distinct character (Polaskova et al., 2008; Ebeler and Thorngate 2009; Gonzalez-Barreiro et al. 2015). Generally, the overall chemical composition of different grape cultivars is similar; however, distinct flavour and aroma differences are clearly observed. This is because the aroma active compounds and precursors are available at different concentrations in each grape cultivar (Delfini and Bardi, 1993; Polaskova et al., 2008). During alcoholic fermentation, yeast synthesises *de novo* aroma active compounds and convert odourless aroma precursors available in grape must into aroma active compounds (Hernández-Orte et al. 2002; Swiegers et al. 2005; Bartowsky and Pretorius 2009; Hart et al., 2017). In fact, some compounds in grape must can only be converted by certain yeast strains into aroma active compounds (Romano et al., 2003). This implies that some yeast strains perform better (*i.e.* enhanced fermentation and varietal characteristic) in one cultivar as compared to another. Thus, it is important to select wine yeasts that will be able to enhance

and complement the distinct character of each grape cultivar. Since climate change has been shown to decrease the aroma profiles of wines (Jagella and Grosch, 1999; Mozell and Thach, 2014), it is important to investigate yeasts that have the ability to augment varietal aromatic characteristics.

Yeasts use two mechanisms to influence the aroma of wine: firstly, by converting odourless grape must precursors into aroma active compounds through enzymatic activity, secondly by the *de novo* synthesis of primary- (ethanol, glycerol, acetic acid and acetaldehyde) and secondary metabolites (esters, higher alcohols, fatty acids acids) (Fleet, 2003). Extensive research has focused on viticultural practices to modulate varietal aromas, whereas limited research has been done on the influence of yeast on varietal aroma. This is due to the general consensus in literature that varietal aromas cannot be influenced by the yeast strain as these compounds (methoxypyrazines, rotundone, and C_{13} norisoprenoids) are directly extracted from the grape skins. This is debatable, as the compounds present in wine interact to show synergistic and antagonistic responses (Polaskova et al., 2008; Von Mollendorff, 2013). This signifies that the varietal aromas can either be enhanced or suppressed by the fermentation bouquet and the released aroma precursors. The use of *S. cerevisiae* to produce wines with different styles has been a research focus for many years (Rapp 1998; Mateo et al., 2001; Dubourdieu et al., 2006; Sumby et al., 2009; Barrajón et al., 2011). Based on these studies it can be suggested that the winemaker can tailor the wines to be either fruity or vegetative by selecting specific yeast strains to conduct alcoholic fermentation.

The aroma of wine is essential as it gives the wine its character and it is a key determinant with regards to wine quality (Vilanova and Sieiro, 2006; Vilanova et al., 2007). Aroma is also an important factor as it is used to differentiate between different wines and wine styles (Swiegers et al., 2005). Wine aroma originates from both the yeast strain selected to conduct the fermentation and the grape cultivar. The grape berry is comprised of free volatile and bound non-volatile compounds, which are responsible for the primary aroma of wine also known as varietal aroma (Swiegers et al., 2005). The non-volatile compounds are aroma inactive precursors, which may be converted to aroma active compounds during wine making, whereas the free volatiles are directly extracted from the grape skin (Villena et al. 2006). Only

a few of the free volatile compounds have been identified as aroma active compounds such as monoterpenes (Rapp and Mandely, 1986), C_{13} norisoprenoids (Winterhalter and Rouseff, 2002), volatile sulphur compounds (Darriet et al., 1995; Tominaga et al., 1996, 1998a), methoxypyrazines (Allen et al., 1991) and rotundone (Wood et al., 2008). The bound precursors are available in the grape must as aroma inactive compounds bound to cysteine (Tominaga et al., 1998b; Thibon et al., 2010), glutathione (Peyrot Des Gachons et al., 2002) and glycoside conjugates (Park et al., 1991) which can be converted to aroma active compounds by enzymatic activity or acid hydrolysis (Styger et al., 2011). Acid hydrolysis may negatively alter the intrinsic varietal aroma of the wine, thus enzymatic hydrolysis is the preferred method to enhance the varietal aroma of wine (Hernandez-Orte et al., 2009).

It is well documented that *S. cerevisiae* can be used to modify wine styles since this yeast greatly affects both the fermentation and the sensory properties of the finished wine. Wine yeast proteins are responsible for these features, thus proteomic analysis may be used to select wine yeast that produces good quality wines (Trabalzini et al., 2003). The use of multiple omics approaches is encouraged to get a clear reflection of the sensory profile of the wine; therefore, metabolomics is usually used with proteomics. The study of metabolites enables researchers to characterise complex phenotypes such as the aromas perceived in wine (Rossouw and Bauer, 2009). The aim of this study is thus to investigate the influence of a natural wine yeast strain ARC Nvbij 6 (*Saccharomyces cerevisiae*) on typical red wine production by utilising proteomic and metabolomic tools. This will enable wine makers to tailor specific wine styles with enhanced varietal aromas.

1.2 AIMS AND OBJECTIVES

The specific aims of this study through to:

1. Compare an experimental dried *Saccharomyces cerevisiae* strain (ARC Nvbij 6) to two different commercial reference *Saccharomyces cerevisiae* strains (MERIT and WE372) to establish whether the experimental yeast produces wine equal or better in quality than the commercial yeast strains.

Aims were achieved by the following objectives:

One experimental dried *Saccharomyces cerevisiae* strain and two commercial reference yeast strains (MERIT and WE372) were used as monocultures to ferment must from three cultivars (Merlot, Cabernet Sauvignon, and Shiraz) in order to:

1. Investigate protein expression of wine yeast strains (ARC Nvbij 6, MERIT, and WE372).
2. Analyse metabolites released during alcoholic fermentation.
3. Conduct sensory and chemical analysis.
4. Establish correlation between various data generated.

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Chapter 2

Literature Review

Influence of *Saccharomyces cerevisiae* on red wine aroma and flavour

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CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Climate change has a worldwide impact on the chemical composition of wine grapes, and the resultant wines produced from these grapes (Jagella and Grosch, 1999; Mozell and Thach, 2014.). Wine quality depends on aroma and flavour, which originates from the wine chemical composition (Louw et al., 2010; Hart et al., 2016). Temperature variations, either too cold or too warm, were previously reported to have a detrimental effect on the wine quality. Vines located (cultivated) in colder climatic regions tend to produce grapes with sub-optimal ripening, resulting in wines with higher acetic acid, lower sugar and mediocre flavours, characterised by dominant vegetative aromas and flavours which compromises the wine quality (Roujou de Boubee et al., 2002; Sansti, 2011). The other extreme, high temperatures, is also detrimental which in viticultural areas results in low acetic acid, high sugar, high alcohol and cooked vegetative aromas and flavours (Sansti, 2011). Both extremes render the wine less fruity. In a quest to preserve the fruitiness in wine, yeast strains that can enhance fruity aromas are sought-after.

S. cerevisiae synthesises a diverse range of aroma enhancing metabolites during alcoholic fermentation, which are responsible for the distinct flavours of alcoholic beverages such as beer and wine (Romano et al., 2003; Swiegers and Pretorius, 2005; Ciani et al., 2010; Saerens et al., 2010). Even though these metabolites are present at very low concentrations in the wine, their concentrations differentiate the aroma profiles of these alcoholic beverages (Cordente et al., 2007). The yeast strains release aroma-active compounds from the aroma-inactive compounds present in the grape must and further synthesises other aroma active compounds through amino and fatty acid metabolism (Lambrechts and Pretorius, 2000; Styger et al., 2011). Previous studies have shown that, in addition to the grape cultivar, the concentrations of aroma active compounds also depend on the specific wine yeast used to carry out alcoholic fermentation (Rossouw et al., 2008; Styger et al., 2011).

Production of varietal aromatic wine using grapes originating from *Vitis vinifera* has progressed extensively (Thomas et al. 1993; Bowers et al. 1999). The intricate sensorial profile

of wine is the result of a large variety of volatile compounds. Nonetheless, few compounds were identified as aroma impact compounds, pertaining to distinct varietal aroma nuances. These compounds include, amongst others, methoxypyrazines in Cabernet Sauvignon, rotundone in Shiraz and thiols in aforementioned cultivars as well as Merlot. The lowest metabolite concentration detected by the taste buds is referred to as the sensory threshold, and those detected by the nose is referred to as the aroma thresholds (Meilgaard et al., 2007; Jackson, 2016). The aroma thresholds of different aroma active compounds differ significantly, and was also shown to influence the effect of other aroma compounds and consequently the wine style and varietal profile (Guth, 1997; Francis and Newton, 2005). Particularly, a compound *e.g.* volatile thiol present in a wine at levels close to its aroma threshold will most probably contribute to the varietal aroma, unless other aroma compounds such as methoxypyrazines are present at levels higher than its aroma threshold to mask its effect. Impact compounds need only be present at low levels to have an impact on the aroma and flavour of wines as they have low aroma detection thresholds, whereas other compounds although present at higher conncetrations, might not even contribute to the aroma and flavour of the wine. This is as a result of high aroma detection thresholds (Von Mollendorff, 2013). The varietal character of a wine is known as the typical aromas and flavour generally ascribed to a specific grape cultivar (Hart et al., 2016; 2017a). The compounds from the grape cultivar contributes to wine varietal character, referred to as true cultivar aroma, and are produced from precursors found at different concentrations in grapes (Polaskova et al., 2008). The overall composition of different grape cultivars is more or less the same per cultivar. However, distinct flavour and aroma differences are clearly observed because of the yeast strain used to carry out alcoholic fermentation. These aromas and flavours are referred to as fermentation bouquet.

2.2 THE INFLUENCE OF YEAST ON WINE AROMA AND FLAVOUR

Modern day winemaking involves direct inoculation of active dried wine yeast (ADWY), which are primarily *S. cerevisiae* strains, into grape must (Suárez-Lepe and Morata, 2012) to ensure fast, predictable and reproducible fermentations as well as better final product quality

(Pretorius, 2000). The ADWY inoculum (yeast strain) must meet several criteria such as the ability to rapidly complete fermentation, withstand harsh conditions and synthesise desired aroma compounds etc. (Degre, 1993; de Nobel et al., 2001; Zuzuarregui and Del Olmo, 2004; Zuzuarregui et al., 2006). Therefore, different yeast strains can be used to attain a certain flavour/aroma profile due to differences in aroma compounds production between strains.

The wine yeast uses two mechanisms for the production of wine aroma compounds. Firstly, the wine yeast secretes enzymes *e.g.* β-lyase that is required to cleave the carbon-sulphur bond of the odourless cysteine conjugate present in the grape must, thereby releasing the aromatic volatile thiols (Hernandez-Orte et al., 2009; Marullo and Dubourdieu 2010; Styger et al., 2011) (Fig. 1).

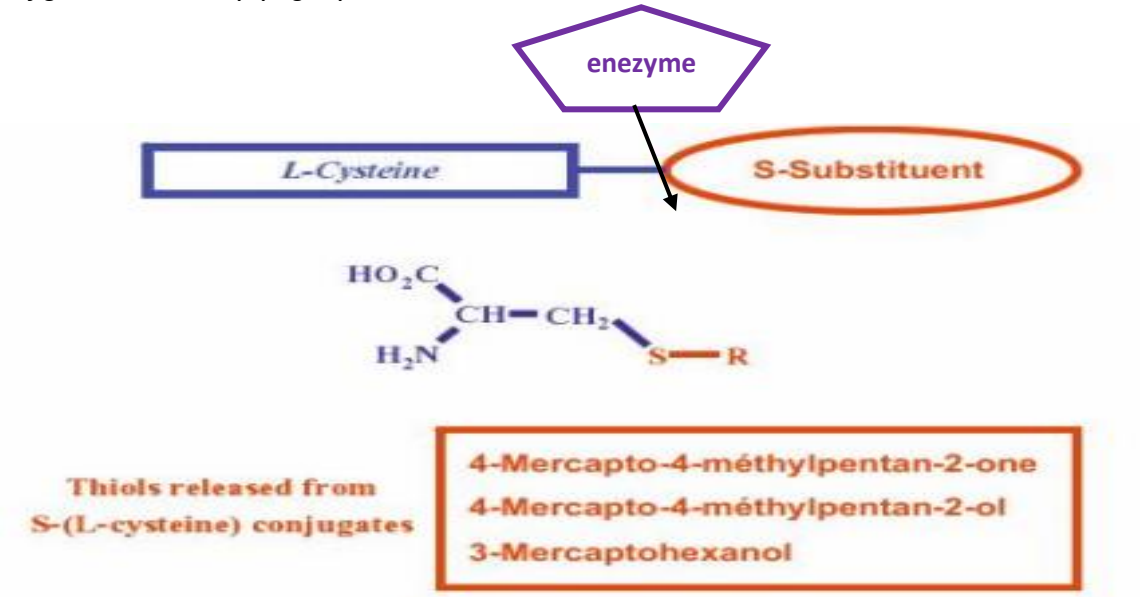

Figure 1. Wine yeast strain derived enzyme are cleaved at the carbon-sulphur bond of the cysteine conjugate to release the aromatic volatile thiols (adapted from Ugliano, 2009).

Secondly, the yeast produces *de novo* secondary metabolites during fermentation that also contributes to the aroma profile of wine (Fleet, 2003; Marullo and Dubourdieu, 2010; Styger et al., 2011) (Fig. 2). In fact, some precursor molecules present in grape must can only be metabolised by a select few yeast strains into aroma active compounds (Romano et al., 2003), and therefore some yeast strains will perform better in one cultivar compared to another.

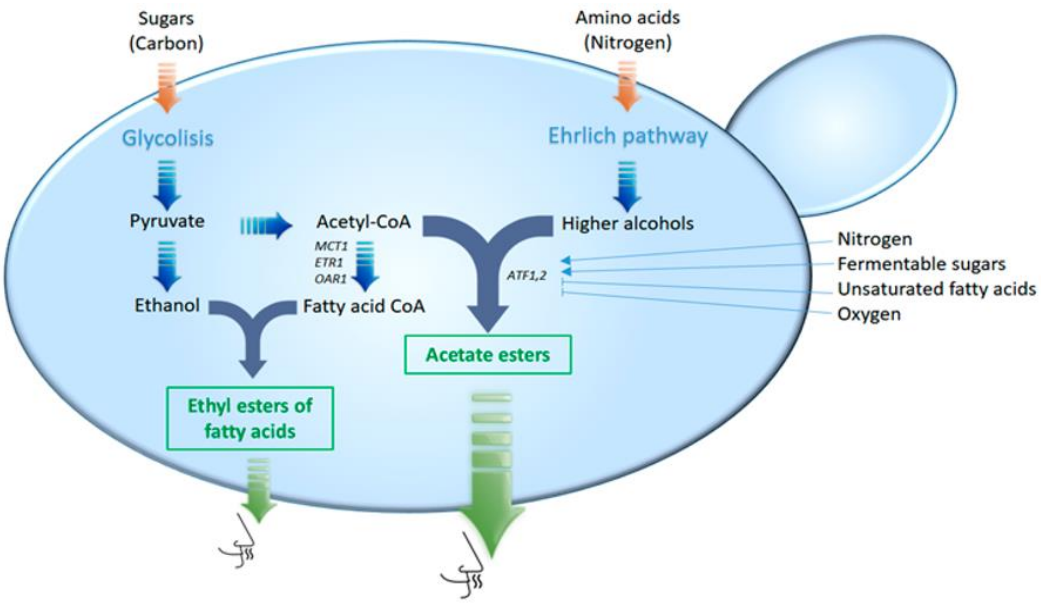

Figure 2. *De novo* synthesis of aroma compounds by wine yeast strains during alcoholic fermentation (adapted from Belda et al., 2017).

Walsh et al. (2006) investigated the aroma profiles of wines produced from the same Shiraz grape must, fermented with different yeast strains *i.e.* AWRI 796 and Maurivin BP 725. Sensory evaluation indicated that the resulting wines displayed differential wine aroma profiles. One yeast strain (AWRI 796) produced a fruitier wine with enhanced black berry and plum aromas, whereas the other yeast strain (Maurivin BP 725) produced a more peppery wine with enhanced spicy and black pepper aromas (Fig. 3). Although yeast strains have no direct effect on rotundone levels by synthesising or modifying the compound, it does have an indirect impact on the perception of the pepper aroma by masking it through the production of secondary aroma active compounds associated with *e.g.* fruity aromas. Thus, the choice of yeast will contribute in either enhancing or masking the peppery aroma and it is imperative to select wine yeast that will be able to enhance the distinct character of each grape cultivar.

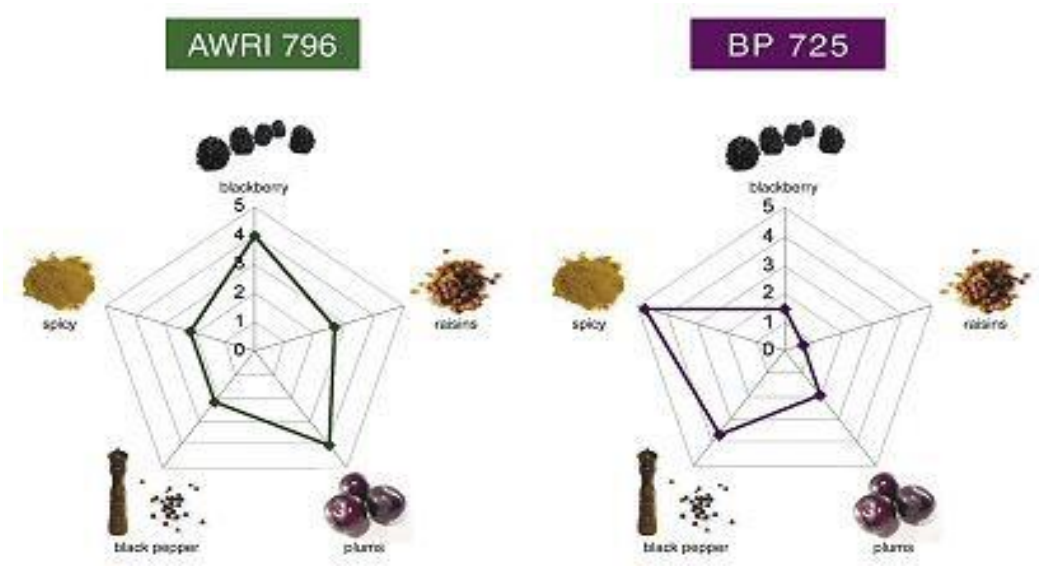

Figure 3. Different aroma profiles displayed by different yeast strains used to ferment the same Shiraz grape must (adapted from Walsh et al., 2006).

2.2.1 The influence of yeast on spicy and vegetative aroma

2.2.1.1 Rotundone

The compound responsible for the peppery aroma perceived in some red wines, especially Shiraz was a mystery, until Wood et al. (2008) unravelled this mystery in a quest to identify the aroma compound(s) responsible for this specific aroma (Table 1). Subsequently, rotundone was identified as the compound responsible for this peppery aroma, which is the same compound present in abundance in *Piper nigrum* better known as black pepper. Rotundone was reported to be synthesised by the grapevine and is located in grape skins and berries (Siebert et al., 2010). The levels of rotundone can be affected by the grape cultivar, wine region and climatic conditions. Thus, the notion that rotundone levels in grapes can be regulated using viticultural practices has emerged (Caputi et al., 2011). However, a yeast strain with the ability to produce higher levels of compounds associated with fruit aromas can somehow mask the peppery aroma in final wines. Therefore, the influence of the yeast inoculum on the wine aroma profile should not be taken for granted. It is noteworthy that the masking effect of the yeast starter culture on rotundone levels has, to our knowledge, never been investigated as these

compounds are grape-derived and the yeast does not change it during fermentation. Nonetheless, this aspect warrants future investigation.

2.2.1.2 Methoxypyrazine

Grape derived compounds,such as methoxypyrazines, are the main aroma contributors pertaining to green and vegetative aromas and flavours *e.g.* green pepper perceived in final wines

(Table 1) (Marais, 1994; Lapalus, 2016). Consequently, this compound has also been reported to be in abundance in green bell peppers. As methoxypyrazines are heat and light sensitive, the warmer climatic conditions in the South African wine regions were shown to negatively affect methoxypyrazine levels during grape ripening (Treurnicht, 2011). Cooler climatic conditions on the other hand are favourable for methoxypyrazine production, which will result in wines with a more green and vegetative aromatic character. Furthermore, viticultural practises such as leaf removal were reported to affect methozypyrazine levels (Swiegers et al., 2006).

The effect of yeast on methoxypyraine levels remain a topic of controversy as Sala et al. (2004) and Lund et al. (2009) made contradictory observations. Sala et al. (2004) reported that the methozypyrazine levels during the initial phases of fermentation differed compared to the end of fermentation, whilst Lund et al. (2009) reported that methoxypyrazine levels did not differ. Another study disagreeing with Lund et al. (2009) reported that the yeast strain had an effect on methozypyrazine levels (2-isobutyl-3-methoxypyrazine), albeit negligible (Marais et al., 2001). A study which investigated whether 2-isobutyl-3-methoxypyrazine levels in Cabernet Sauvignon could be modulated by the yeast starter culture, observed that the yeast strains were able to mask the vegetative aromas by enhancing other aromas (Pickering et al., 2008). Van Wyngaard et al. (2014) also investigated the interaction between methozypyrazines (incurs vegetative aroma) and volatile thiols (incurs tropical fruit aroma), and reported that these compounds had an antagonistic effect on each other at certain concentrations. In other words, higher volatile thiol concentrations will supress

the green aromas as a result of enhanced tropical fruit aromas perceived. In conclusion, no mechanism for the synthesis of methoxypyrazines by *Saccharomyces* yeast has ever been reported. However, as there is evidence in favour and against this notion, an in-depth investigation into the effect of yeast strains on methozypyrazine levels, and the mechanism used by the yeast to synthesis this compound, can in future be undertaken.

2.2.2 The influence of yeast on fruity aroma

2.2.2.1 Esters

Esters are yeast-derived chemical compounds that impart fruity and fresh aromas in wines, and are classified as acetate esters and ethyl esters, respectively (Table 1) (Rossouw et al., 2008; Styger et al., 2011). Ethyl esters are comprised of an alcohol group and an acid group which is a medium chain fatty acid (MCFA), whilst acetate esters are comprised of an acid group and an alcohol group *viz.* ethanol or higher alcohol, produced during amino acid metabolism. Acetate esters are generally associated with aromas such as banana, honey and roses while ethyl esters specifically attribute an apple-like aroma to the wines (Saerens et al., 2008). The predominant esters found in wine are alcohol acetates and C4–C10 fatty acid ethyl esters (Schreier, 1979). The typical fruity aromas perceived in wine are mainly due to the following esters; hexyl acetate, ethyl caproate, ethyl caprylate isoamyl acetate and 2-phenylethyl acetate (Lambrechts and Pretorius, 2000; Swiegers et al., 2005; Swiegers and Pretorius, 2005). A study conducted by Plata et al (2003) investigated the ability of several wine yeast strains to synthesise ethyl acetate and isoamyl acetate, and reported that the formation of these two compounds differed between wine yeast strains.

2.2.2.2 Thiols

The volatile thiol compounds *viz.* 4-mercapto-4-methylpentan-2-one (4MMP) and 3-mercaptohexan-l-ol (3MH) were shown to have a significant effect on the varietal aroma of wine, especially in Sauvignon blanc wines (Table 1) (Holt et al., 2011; Roncoroni et al., 2011).

These compounds are bound non-volatiles originating from the grape berries as cysteine conjugates which are released during fermentation by the wine yeast through enzymatic activity (Swiegers et al., 2005; Swiegers et al., 2007; Holt et al., 2011). Another thiol namely, 3-mercaptohexyl acetate (3MHA) has no cysteine conjugate precursor and is formed during alcoholic fermentation from the thiol 3MH by wine yeast through esterification with acetic acid. In addition, yeast-derived alcohol acetyltransferase was reported to be the principal enzyme involved in the formation of 3MHA (Swiegers et al., 2005; Swiegers et al., 2007). The concentration of the volatile thiols is significantly lower in the grape must compared to the bound-volatiles, and it was reported that they are nearly non-existent in grape berries and/or juice (Capone et al., 2011).

Table 1. Compounds that affect the varietal aroma of wine.

Metabolite	*OPT in water	Origin	Aroma	References
Methozypyrazine ([a]IBMP)	2 ng/L	Originate in grapes	Bell pepper Green beans Herbaceous	Buttery et al., 1969
([b]SBMP)	1 ng/L		Asparagus Earthy	Dubourdieu et al., 2006
([c]IPMP)	2 ng/L		Pea Asparagus	Marais, 1994
Thiols		Precursors in grape berries converted during fermentation by wine yeast		Darriet et al. (1995).
[d]4MMP	0.8 ng/L		Box tree Black currant	
[e]3MHA	60 ng/L			Tominaga et al. (1998); Dubourdieu et al. (2006).
[f]3MH	4.2 ng/L		Box tree Passionfruit Tropical guava	Tominaga et al. (1996); Dubourdieu et al. (2006)
Esters	0.2-7.5	Yeast metabolism during fermentation	Fruity Floral Rose oil Perfume	Swiegers and Pretorius (2005)
Rodundone	16 ng/L	Originate in grapes	Black pepper	Wood et al. (2008)
Monoterpenes	170 ng/L	Originate in grapes	Fruity/floral aromas	Marais (1994)

*Olfactory perception threshold
[a]2-isobutyl-3-methoxypyrazine
[b]2-sec-butyl-3-methoxypyrazine
[c]2-isopropyl-3-methoxypyrazine
[d]4-mercapto-4-methylpentan-2-one
[e]3-mercaptohexyl acetate
[f]3-mercaptohexan-l-ol

In fact, the majority of volatile thiols are synthesised during alcoholic fermentation by the wine yeast strain inoculum from their bound non-volatile cysteine precursors present in grape berries/must (Tominaga et al., 1998; Peyrot des Gachons et al., 2002; Swiegers et al., 2005; Dubourdieu et al., 2006; Swiegers et al., 2009). Therefore, yeast strains that are able to convert these bound non-volatiles to aromatic volatiles are sought after. It can be said that without these thiol-releasing yeast strains, the modulation and enhancement of varietal aromas of final wines associated with these compounds of the wine would not be achieved. Most studies focusing on volatile thiols were mostly conducted on Sauvignon blanc wines, and it has been established that these compounds significantly impact the varietal aroma of the wines produced by this specific cultivar (Dubourdieu et al., 2006; Lund et al., 2009; Hart et al., 2017a). Aroma and flavour nuances associated with aforementioned volatile thiols include grapefruit, blackcurrant and passion fruit etc. (Table 1) (Tominaga et al., 1998; Rantz, 2001). Although thiols have been detected in Merlot and Cabernet Sauvignon (Murat et al., 2001), very little research have been conducted to investigate the effect of these compounds in these red wine varieties. A recent study conducted by Rigou et al. (2014) investigated the effect of 4MMP on the characteristic blackcurrant aroma perceived in red wines and concluded that this compound enhances the blackcurrant aroma. It is evident that yeast inoculum is very important as the release of volatile thiols is strain dependent, and will influence the final wine aroma and flavour (Coetzee and Johaness, 2012).

2.3 ANALYSES OF METABOLITES (COMPOUNDS) AND SENSORY EVALUATION

Several methods amongst others, gas chromatography-mass spectrometry (GC-MS) and gas chromatography-olfactory (GC-O) can be deployed for the measurement of aroma active compounds that contribute to wine aroma and flavour (Lawrence et al., 2012). Quantification of metabolites are dependent on two factors namely, the physicochemical properties of the metabolites and the levels (concentration) of the metabolites in the matrix to be analysed (Lawrence et al., 2012). Furthermore, metabolite quantification can be categorised as either targeted or non-targeted quantification. The difference between these two approaches is that

with targeted quantification, sought-after compounds are measured and identified (Ramautar et al., 2006), whereas the untargeted approach focuses on determining the presence of as many metabolites as possible (Monton and Soga, 2007).

Metabolites present in wine vary in concentrations, hence different protocols *e.g.* solid phase extraction (SPE) and solid phase micro-extraction (SPME), for the extraction and concentration of these compounds can be deployed as listed in Table 2 (Castro et al., 2008). Thereafter, detectors play a fundamental role, in conjunction with gas chromatography (GC), for the quantification and identification of aforementioned wine compounds. Various detectors *viz.* flame ionization detectors (FID), mass spectrometry (MS) and olfatoctometry (GC-O) can be deployed in this regard, all of which differs in detection limits, specificity and linear ranges (Pino and Queris, 2011a; 2011b). Flame ionization detectors (FID) is the most cost-effective, and thus the most used detector for the analysis of aroma active compounds (Palomero et al., 2009; Louw et al., 2010). Even though MS is more expensive than FID, it has been used considerably for the analysis of aroma active compounds in wine, especially for compounds that cannot be detected using FID (Dziadas and Jelen, 2010; Pino and Queris, 2011a; 2011b).

Table 2. Detectors used in conjunction with gas chromatography (GC) to determine volatile compounds in wine.

Detectors	Identification mode and Principle	Advantages	References
Flame ionization detectors (FID)	Analytes ionised in hydrogen flame Identify analytes based on conductivity between two electrodes Response results from conductivity between two electrodes	Wide linear range High sensitivity Less expensive than MS	Louw et al., 2010; Pino and Queris, 2011
Mass spectrometry (MS)	Analytes blasted by electrons Identify analytes by mass spectra	Very specific and sensitive	Gil et al., 2006.
Olfactometry (O)	Combination of human and electronic responses Linking aromas to human perception	Odour detection value determined	Mayol and Acree, 2001

Sensory evaluation, especially descriptive analysis in conjunction with GC-based analyses have become increasingly important to determine aromatic characteristics exerted by volatile compounds present in final wine (Lapalus, 2016). In addition, several statistical analyses such as principal component analysis (PCA), partial least squares (PLS) and multiple factor analysis (MFA) are used to determine whether volatile compounds detected have a positive or negative correlation with aromatic characteristics established using descriptive sensory analysis (Noble and Ebeler, 2002; Francis and Newton, 2005).

2.4 ROLE OF YEAST PROTEINS IN WINE AROMA

Several researchers previously reported on the important contribution of wine yeasts on the final wine organoleptic quality (Callejon et. al., 2010; King et al., 2010; Sumby et al., 2010; Medina et al., 2013). This contribution stems from the production of certain metabolites such as ethanol, glycerol and acetic acid to the more intricate contributions such as the ability to prevent protein haze formation and complex aroma profiles (Lubbers et al., 1994a; Lubbers et

al., 1994b; Belancic et al., 2003; Andorrà et al., 2010). Wine yeast-derived proteins were also reported to be involved in these features displayed by the wine yeast, thereby making proteomics a possible tool to evaluate and select optimal wine yeast strains (Trabalzini et al., 2003).

Most studies conducted on wine yeast proteins focused on the role of the enzyme *β*-lyase in the enhancement of tropical fruit aromas due to the released volatile thiols associated with said aromas (Thomas and Surdin-Kerjan, 1997; Swiegers et al., 2006, Swiegers et al., 2007). The effect of *β*-glucosidases on wine aroma was also previously reported (Blasco et al., 2006), whilst other studies focused on the role of mannoproteins on haze prevention in wine (Dupin et al., 2000; Gonzalez-Ramos et al., 2008; Ndlovu et al., 2012), and foam formation in sparkling wines (Fukui and Yokotsuka, 2003; Charpentier et al., 2004; Cilindre et al., 2008; Blasco et al., 2011).

Muñoz-Bernal et al. (2016) investigated the effect of temperature on the protein profile (proteome) of *Saccharomyces bayanus var. uvarum* during fermention of grape must at 14 °C and 25 °C, respectively. The authors suggested using differentially expressed proteins as biomarkers to select new wine yeast strains with the ability to ferment at low temperatures. Another study conducted by Moreno-García et al. (2015), one of the very few studies if not the only study thus far, compared the proteome and exometabolome of a *S. cerevisiae* flor yeast strain grown under two different conditions, *viz.* biofilm formation and no biofilm formation. The authors established an association between differentially expressed proteins and aroma active compounds produced by the *S. cerevisiae* flor yeast. This observation, therefore, suggests that the identification of protein biomarkers associated with metabolites and ultimately the aroma of the wine is possible, which is one of the objectives of this study. Most of the protein studies investigated the influence of proteins on haze formation and prevention as well as foam formation in sparkling wines with very few studies focusing on the yeast proteome, and how it may influence wine properties. In particular, the relationship between wine yeast-derived proteins and metabolites produced during fermentation and how it might contribute to the sensory profile of wines remains unexplored. This avenue of wine yeast and wine quality warrants further investigation.

2.4.1 Analyses of the yeast proteome

The objective of proteomics involves the study of all expressed and regulated proteins within an organism (Perez-Ortın and Garcıa-Martınez, 2011). There is one major drawback when it comes to genomics and transcriptomics both are not able to directly reveal gene function as messenger RNA (mRNA) only conveys the genetic blue print but is not the actual functional molecule (Carpentier et al., 2008). Furthermore, weak correlations between mRNA and protein levels were found in several studies, due to post transcriptional modification of expressed proteins (Carpentier et al., 2008; Perez-Ortın and Garcıa-Martınez, 2011). Post transcriptional processes such as RNA-splicing and poly-adenylation was shown to result in more than one functional product from the same gene (Gingold and Pilpel, 2011). These gene products are proteins, the actual functional molecules (final effectors), and the closest biological level to the metabolome. It can be envisaged that proteomics will provide a better reflection of the organism's (*e.g.* wine yeast) phenotype (*e.g.* release of wine aroma enhancing metabolites) than transcriptomics (Perez-Ortın and Garcıa-Martınez, 2011).

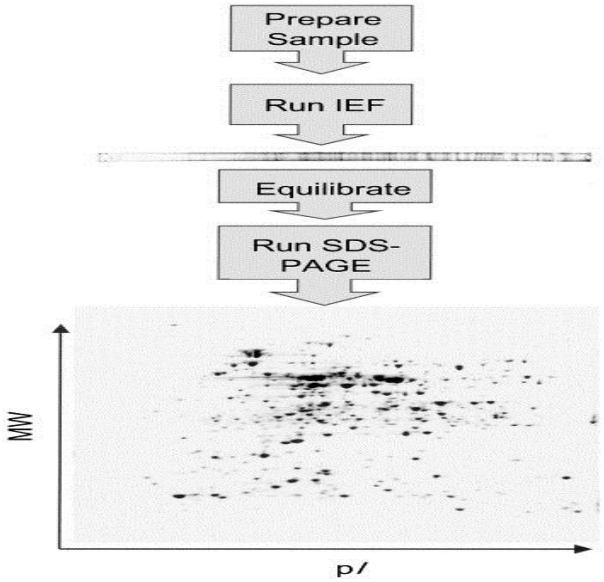

Figure 4. Schematic depiction of two-dimensional (2D) poly acrylamide gel electrophoresis (PAGE) (adapted from Garfin, 2003).

The prospect of proteomics to identify differentially expressed proteins under different physiological conditions is fascinating for biotechnologists, as it can be used for the

identification of biomarkers (Basak et al., 2016; Chang et al., 2017; Kupfer et al., 2017). Conventionally, one dimensional (1D) and two-dimensional (2D) poly acrylamide gel electrophoresis (PAGE) are deployed in this regard, as they are inexpensive (Fig. 4) (Abdallah et al., 2012). Briefly, 1D PAGE commonly referred to as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins based on molecular weight (kDa) (Gallagher, 2012). However, proteins are amphoteric molecules, as they can either have a negative, positive or zero net charge. Thus, an ionic detergent, namely SDS with the ability to denature proteins and form a negatively charged protein/SDS complex is used in this regard (Chevalier, 2010). The quantity of SDS bound to the protein usually equates to the mass of the protein, hence all negatively charged proteins are separated strictly based on molecular mass (Hames, 1998; Chevalier, 2010), enabling proteins to travel to the positive anode when placed within an electric field (Gallagher, 2012).

The 2D-PAGE, on the other hand, separates proteins based on two dimensions namely, molecular weight and isoelectric points which in essence is the pH value at which the protein has a net charge of zero. (Garfin, 2003, Hart et al., 2017a). Subsequently, the use of 2D PAGE became popular, as it provides useful information pertaining to expressed proteins such as the molecular size, isoelectric point (pI) (Klose, 1975.; O'Farrell, 1975; Gallagher, 2012). Currently, this is a standard gel-based method used to investigate the proteome of a biological sample (Garfin, 2003) and even today the biology community continues to use it for yeast expression studies (Mostert et al.,2013; Muñoz-Bernal et al., 2016; Szopinska et al., 2016). In addition, proteins separated with 2D PAGE are stable and long-term storage can be achieved preceding further analysis (Görg et al., 2004; Rabilloud et al., 2010). Like any other analytic method, this method too has limitations which include difficulty in reproducibility as well as the possibility of missing hydrophobic and low abundance proteins, as they are under-represented (Rossignol et al., 2009; Pfeffer et al., 2012; Vanz et al., 2012). Nonetheless, 2D- PAGE remains a useful method to separate complex protein mixtures, and is often used in conjunction with in-gel tryptic digestion and sophisticated mass spectrometry for protein identification (Fig. 5) (O'Farrell, 1975; Lund et al., 1996; Rossignol et al., 2009). Furthermore, most yeast protein expression studies deploy conventional 2D PAGE

(Kobi et al., 2001; Vido et al.,2001; Trabalzini et al.,2003; Brejning et al., 2005; Rossignol et al.,2009).

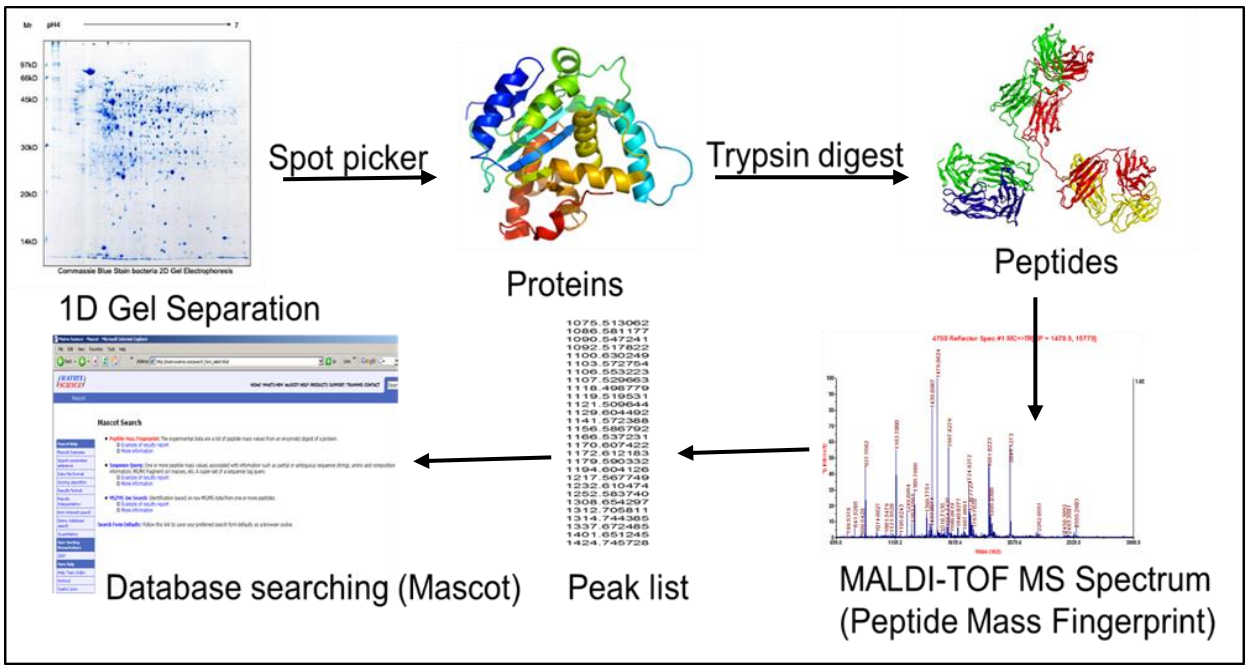

Figure 5. The steps followed to identify proteins. Following Two-dimensional PAGE the images are analysed using a specific software.

Expressed proteins are digested with trypsin and analysed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-THOF/MS) giving rise to a peptide mass fingerprint (PMF). This PMF is then inserted into a protein database for identification purposes. Desorption (vaporisation) and ionisation are the first steps in the MALDI-TOF MS process (Ngara et al., 2012), as a mass spectrum can only be generated when analytes have been vaporised and ionised. Subsequently, solid phase and liquid phase analytes are converted to gas phase ions (Tjernberg, 2005). The process entails embedding of vaporised and ionised analyte in an excess of matrix, which is a weak acid (2, 5-dihydroxy-benzoic acid (DHBA), sinapinic acid (SA) and α-cyano-4-hydroxy-cinnamic acid (CHCA), which absorb strongly at the wavelength of the laser once the latter is beamed onto the matrix (Fig. 6). Subsequently, a strong interaction between the analyte components and the matrix is established (Hillenkamp and PeterKatalinic, 2007).

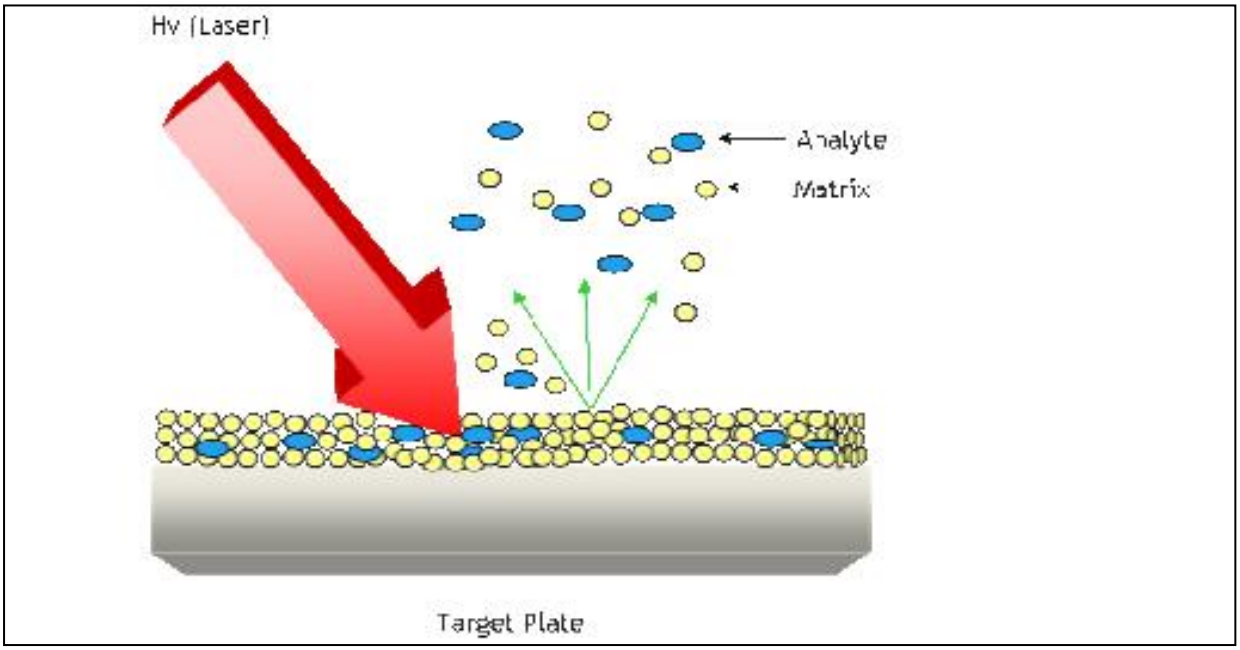

Figure 6. Conversion of analytes to gas phase ions by laser irradiation (Adapted from Wilkins and Lay, 2006).

Sample preparation prior to MALDI-TOF MS involves the successive application of sample and the matrix solution to the MALDI target plate. Thereafter, the sample/matrix mixture (target spot) is air-dried and placed into the mass spectrometer's ion source. Subsequently, the sample/matrix target spot is heated and excited by subjecting it to laser irradiation (Fig. 7). The energy generated by the laser is absorbed by the matrix projecting the sample/matrix mixture in an upward motion as seen in Fig 6. The high vacuum causes desorption (vaporisation), as the mass spectrometer has a high vacuum setting, thus requiring less heat. The matrix serves as a carrier for the analyte transporting it into the gas phase (Wilkins and Lay, 2006, Mootho-Padayachie, 2011).

Time of flight mass spectrometry is the most used analyser for MALDI, as it is affordable (Hillenkamp and Peter-Katalinic, 2007) and very fast, so fast that several repeats can be done to increase accuracy (Wilkins and Lay, 2006). So much that ions are generated in nanoseconds (Hillenkamp and Peter-Katalinic, 2007) when an electric current is applied in the ion source (source region) (Fig. 7). Resultant ions are accelerated to the analyser, where they are separated based on their mass to charge ratio. Subsequently, a mass spectrum is

generated as ions are detected at different time intervals by the detector. It is noteworthy that, no electric current is applied in the analyser, as TOF works on the premise that charge smaller ions will travel faster (shorter time of flight) through the field-free flight than larger ions (Fig. 7) (Twyman, 2004; Wilkins and Lay, 2006). The MALDI TOF/MS also has other applications, as it has been used in several studies to differentiate and characterise yeast strains, although still largely neglected (Qian, et al., 2008, Moothoo-Padayachie, 2011; Gutiérrez et al., 2017). Furthermore, the relationship between expressed wine yeast proteins and metabolites produced during fermentation and the possible effect on the sensory properties of wine is even more neglected and warrants further investigations.

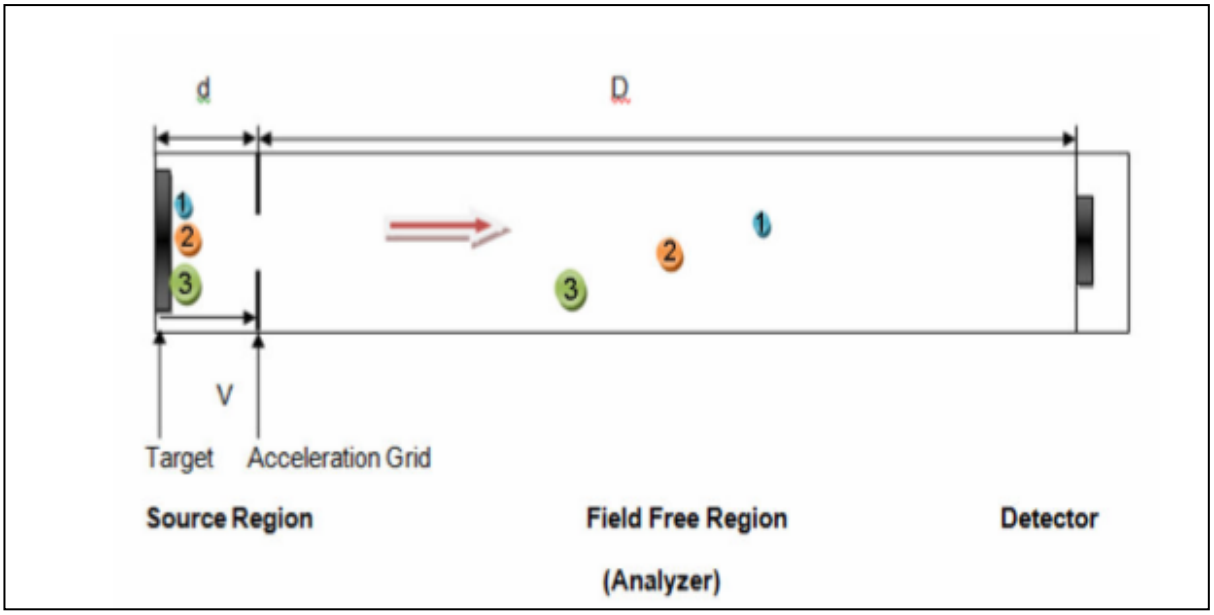

Figure 7. Time of flight mass spectrometry (TOF/MS) is based on how fast the ions (1, 2, and 3) travel from the ion source (d) to the detector after being accelerated by applying an electric current to the source region (d), the smaller ions (1) will travel faster across the field free region (D) than the larger ions (3) reaching the detector first (Adapted from Wilkins and Lay, 2006)

The use of gel-free proteomics (Fig. 8) can be used to overcome problems pertaining to SDS-PAGE and 2D-PAGE mentioned above. Gel free methods use multi-dimensional capillary liquid chromatography (LC) in conjunction with tandem mass spectrometry (MS/MS) to characterise peptides acquired following digestion of the protein extract (sample) with the

proteolytic enzyme *i.e.* trypsin (Baggerman et al., 2005; Xie et al., 2011). Therefore, the resultant tryptic digests (peptides) are characterised as opposed to the actual proteins. However, LC-MS based proteomics is very expensive and requires sophisticated equipment, whereas gel-based proteomics remains relatively cheap and reliable as mentioned above.

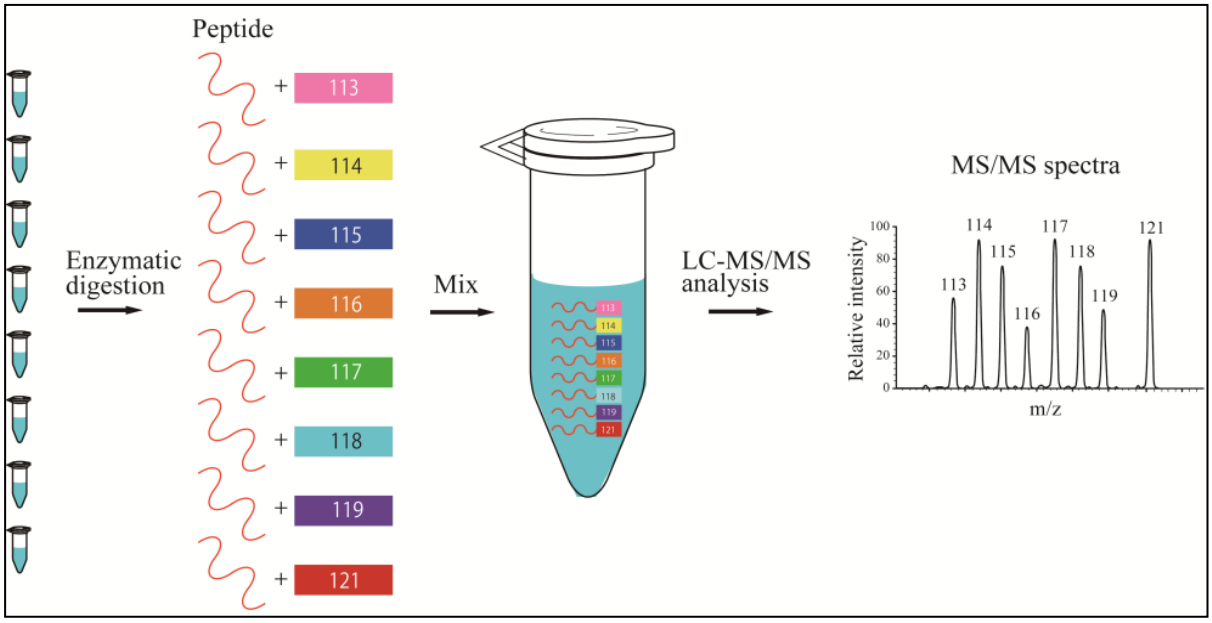

Figure 8. A gel free method (LC-MS/MS) for peptide quantitation (Kozuka-Hata et al., 2013).

2.5 CONCLUDING REMARKS

This review highlights the importance of wine yeast selection by emphasising on the significant role wine yeast play in wine aroma and flavour. It also presents proteomics as a possible tool in the wine yeast selection process. Wine yeast selection is an important part of winemaking (Moothoo-Padayachie et al., 2013); as these microbes, can produce wine from relatively 'neutral' grape juice and tailor wines into a specific style (Richter et al. 2013; Swiegers et al. 2009). Currently, researchers continue to develop new wine yeast as winemakers seek new ways to enhance and diversify their wines, due to increasing competition in the wine industry. The selection of new wine yeast strains is very time-consuming and costly (Usbeck et al. 2014). Thus, the use of proteomics has emerged as a potential tool to rapidly select yeast strains with sought-after traits (Hart et al., 2017b). However

little research thus far has focused on this unchartered field of wine yeast selection and development. Recent studies conducted by Moothoo-Padayachie et al. (2013), Usbeck et al. (2014); Hart et al. (2016) and Gutiérrez et al. (2017) investigated and successfully used MALDI TOF MS/MS as a yeast differentiation tool.

Furthermore, Usbeck et al. (2014) also investigated variations in the enzymatic profiles of various yeast strains based on their peptide profiles. The current study takes it a step further, in addition to investigating differential protein expression, metabolite analysis is also conducted. We hypothesise that different yeast strains will produce wines with different chemical and sensory profiles due to different metabolites produced during fermentation, which in turn are instigated by differential expressed proteins.

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Chapter 3

Characterisation and evaluation of wine yeast used for the production of typical varietal red wines

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CHAPTER 3: CHARACTERISATION AND EVALUATION OF WINE YEAST USED FOR THE PRODUCTION OF TYPICAL VARIETAL RED WINES

3.1 ABSTRACT

The selection of the wine yeast strains, belonging to the species *Saccharomyces cerevisiae* is a crucial aspect of the winemaking process, and a key requirement is to complete the fermentation, whilst simultaneously producing varietal and aromatic wines. Wine yeasts, however, have differentiating abilities to release aroma compounds *e.g.* volatile thiols or produce them *e.g.* esters, hence this study was initiated to investigate the influence of a naturally isolated wine yeast strain *i.e.* ARC Nvbij 6 on typical red wine production compared to two commercial references *i.e.* WE372 and MERIT, respectively. Winemaking trials were initiated in Shiraz, Merlot and Cabernet Sauvignon grape cultivars during the 2016 and 2017 vintages, followed by chemical, sensory, proteomic and metabolomic analyses of fermenting and final wines. The yeast strain ARC Nvbij 6 produced Shiraz, Merlot, Cabernet Sauvignon during both vintages, equal and in some instances better than both commercial references, especially pertaining to lower volatile acidity and acetic acid. These compounds impart unpleasant off-odours, which can mask sought-after varietal aromas and flavours. This notion was supported by red wine descriptive sensory evaluations, as the ARC Nvbij 6 strain produced Shiraz, Merlot, and Cabernet Sauvignon wines with varietal traits. Gas chromatography also showed ARC Nvbij 6 to be a better volatile thiol converter (3-mercaptohexan-1-ol [3MH] to 3-mercaptohexyl acetate [3MHA], as both commercial references failed to convert 3MH to 3MHA during one vintage in two cultivars. Both commercial reference strains did, however, produce red wines with higher ester concentrations than the ARC Nvbij 6 strain. Furthermore, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that all yeast strains differentially expressed proteins within given molecular weights. It is envisaged that peptide mass fingerprinting (PMF) in conjunction with matrix-assisted laser desorption ionization with time of flight mass spectrometry (MALDI-TOF MS) will be deployed to characterise specific regulated proteins expressed by the wine yeasts.

Key words: *Saccharomyces cerevisiae* esters, protein, red wine, varietal, volatile thiols

3.2 INTRODUCTION

Red wines are said to have many health benefits such as reduced risk of heart disease, depression and some cancers (Yao et al., 2004; Castello and Tessitore, 2005; Leifert. and Abeywardena, 2008; Shukla and Singh, 2011; Arranz et al., 2012). Some researchers even recommend one to two glasses per day, however within moderation (O'Keefe et al., 2007; Arranz et al., 2012). As much as wine has health benefits, it must also be enjoyable which implies that the wine aroma and flavour has to be intriguing. The wine yeast *S. cerevisiae* is of cardinal importance for the production of wines with specific varietal aromas *viz.* 'strawberry', 'raspberry', 'blackcurrant', 'plum', 'caramel', 'herbaceous and/or vegetative', to 'spicy', and even 'peppery' (Walsh et al., 2006). Furthermore, wine yeasts were reported to be efficient tools to modulate and enhance wine aroma and flavour (Van Breda et al., 2013; Du Plessis et al., 2017).

The grape berry and juice is comprised of free volatile and bound non-volatile compounds (metabolites), which are responsible for the primary sensory attributes of wine often referred to as varietal aroma and flavour (Swiegers et al., 2005; Robinson et al.,2014). The wine yeast strain used for alcoholic fermentation also contributes to varietal aroma and flavour by converting the non-volatile bound compounds present in the grape berries and juice to aromatic volatile compounds during fermentation. In addition, wine yeasts synthesise other aroma active metabolites *e.g.* esters (imparts fruity aroma nuances) often referred to as the "fermentation bouquet" (Coetzee and du Toit, 2011). Thus, wine sensory characteristics originate from grape-derived metabolites (Ebeler and Thorngate 2009; Gonzalez-Barreiro et al. 2015), yeast-synthesised and yeast-released metabolites (Hernández-Orte et al. 2002;Swiegers et al. 2005; Bartowsky and Pretorius 2009; Hart et al., 2017a).

Volatile thiols namely, 4-mercapto-4-methyl-2-pentanone (4MMP), 3-mercapto-1-hexanol (3MH) and 3-mercapto-hexyl acetate (3MHA) were previously reported to be associated with berry aroma, blackcurrant specifically in red wines (Rigou et al., 2013). The above-mentioned volatile thiols are also known as 4-methyl-4-sulfanylpentan-2-one (4MSP), 3-sulfanylhexan-1-ol (3SH), and 3-sulfanylhexyl acetate (3SHA), respectively

(Renault et al., 2016). It was also reported that yeast expressed proteins are involved in the release of these volatile thiols from their bound non-volatile precursors present in grapes during alcoholic fermentation (Moreno-García et al. 2015 and Hart et al., 2017b). It is noteworthy that the volatile composition of a final wine is directly responsible for wine volatile aroma and flavour, and ultimately wine quality. Red wine yeasts with the ability to produce red wines with typical varietal aroma and flavour may have a positive commercial impact, as some sensory attributes are of importance in terms of consumer preference (Lattey et al., 2010). Commercial yeast manufacturers can also incur financial gain, should a commercial wine yeast strain within their portfolio enhance varietal aromas.

As winemaking styles are ever-changing, especially due to climate change, which results in grapes with sub-optimal ripening (Jones et al., 2005), it is important to develop wine yeast strains that adhere to certain criteria in order to enhance typical varietal aromas. The effects of climate change on grape chemical composition has become more apparent as fermentation kinetics, shifts in microbial populations and flavour and aroma properties have been significantly affected. Of importance to the current study is the reduction in varietal precursors and compounds, which results in reduced wine aroma and flavour (De Orduna, 2010).

Development of new wine yeast is a constant field of improvement as wine yeast can be used to diversify wine styles which winemakers are constantly seeking. Secondly, the association between metabolites released by these wine yeasts and the effect on wine organoleptic quality was also investigated. A plethora of studies have confirmed that the chemical composition of wine can be significantly influenced by the yeast strain used to conduct alcoholic fermentations, thus different yeast strains produce wines with distinct flavour and aroma profiles (Miller et al., 2007; Torrens et al. 2008, Bisson and Karpel 2010; Callejon et al. 2010; King et al. 2011; Robinson et al. 2011; Richter et al. 2013). This is attributed to the genetic differences among yeast strains, which has been thoroughly studied (Hauser et al., 2001; Bisson and Karpel 2010; Rossouw et al., 2010; Steenwyk and Rokas, 2011). Surprisingly the workhorses, which are the proteins, are not so well understood in this regard. The proteins expressed during fermentation, their influence on the metabolites produced, and the

subsequent influence on the aroma and flavour perceived in the wine remains unclear. Differential protein expression will be reflected in the phenotype (aroma and flavour) of the wines. Thus, this was one of the objectives of the current study.

Wine yeasts suitable for the production of Shiraz wine in the Paarl region renowned for its premier Shiraz wines and hot summers were also identified as a criterion for red wine yeast development. One *Saccharomyces cerevisiae* strain isolated from Shiraz grapes *i.e.* ARC Nvbij 6 emerged as a potential candidate based on the results of standard chemical, gas chromatographic (GC) and sensory analyses following a series of fermentation trials using Shiraz, Cabernet Sauvignon, and Merlot grapes as reported at the 34th South African Society for Enology and Viticulture (SASEV) International Congress, Allée Bleue, Symondium, South Africa (Hart et al., 2012). However, to date the characterisation of the above-mentioned naturally isolated red wine yeast strain *i.e.* ARC Nvbij 6 during fermentation of Shiraz, Cabernet Sauvignon, and Merlot grapes using proteomic and metabolomic techniques in conjunction with wine chemical and sensory evaluation has not been investigated. Therefore, the aim of this study was to evaluate and compare a naturally isolated wine yeast to commercial red wine yeasts for the production of typical varietal red wines using proteomic and metabolomic analyses tools in conjunction with wine chemical and sensory evaluation.

3.3 MATERIALS AND METHODS

3.3.1 Yeast strains

One *Saccharomyces cerevisiae* wine yeast strain isolated from Shiraz grapes *i.e.* ARC Nvbij 6 conserved in the ARC Infruitec-Nietvoorbij microorganism culture collection (ARC Inf-Nvbij CC) was used in this study. Two commercial red wine yeast strains *i.e.* WE372 (Anchor Yeast, Cape Town, South Africa) and MERIT (Chr. Hansen, Hørsholm, Denmark) were included as references.

3.3.2 Pulsed-field gel electrophoresis (PFGE)/Contour clamped homogeneous electric field (CHEF) DNA karyotyping

DNA karyotyping of yeast strains was conducted according to the embedded agarose procedure described by Hart et al. (2016). Briefly, one gram active dried yeast of starter cultures (ARC Nvbij 6, MERIT and WE372) as well as yeast isolated (one colony) at the end of the alcoholic fermentation, were inoculated into 100mL YPD broth (Biolab, Merck). Cells were harvested from the YPD broth by centrifugation (Beckman Coulter™ Avanti J-25, USA) for 10 min at 8000 rpm. Subsequently, the yeast pellet was washed twice with 10 mL of 10 mM EDTA pH 7.5 and was centrifuged at 4 °C for 10 min at 8000 rpm. The supernatant was discarded and the yeast pellet was resuspended in 3 mL 50 mM EDTA pH 7.5. This yeast suspension was mixed with disruption buffer 1 (1 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA pH 5.8, 0.05% [w/v] *β*-mercapto-ethanol and 0.1% [w/v] lyticase) at a ratio of 1:3. The yeast suspension was mixed with melted (50 °C) gelling solution (1% [w/v] low-melting temperature SeaPlaque® agarose, 0.125 M EDTA pH 7.5) at a ratio of 1:5. The suspension was homogenised by gentle shaking and transferred to sterile petri-dishes and evenly spread and stored at room temperature to gel (solidify). Using a surgical blade, the gelled yeast suspension was aseptically cut into 5 x 10 x 10 mm gel cubes (plugs) and the latter was transferred to a sterile glass container and submerged in disruption buffer 2 (0.45 M EDTA pH 9.0, 10 mM Tris-HCl pH 8.0, 0.05% [w/v] *β*-mercapto-ethanol). Subsequently, the container with the plugs was incubated at 37 °C for 24 hours, whereafter disruption buffer 2 was decanted and replaced with a washing buffer (0.45 M EDTA pH 9.0, 10 mM Tris-HCl pH 8.0, 1% [w/v] Na-N-Lauroylsarcosinate, 0.1% Proteinase K [w/v]). The container with plugs was incubated at 50 °C for 48 hours, whereafter the washing buffer was decanted and replaced with a storage buffer (0.5 M EDTA pH 9) and stored at 4 °C until it was required for pulsed-field gel electrophoresis (PFGE)/contour clamped homogeneous electric field (CHEF) karyotyping. Chromosomal DNA was separated in 0.5X TBE diluted from 10X TBE buffer (121.1 g/L Tris, 51.53 g/L boric acid, and 3.27 g/L EDTA [Sigma-Aldrich, USA]) at 14 °C with pulse-times of 60 seconds for fifteen hours and 90 seconds for 11 hours using clamped homogenous electric

field (CHEF) gel electrophoresis (CHEF-DR II, Bio-Rad Laboratories, Richmond, USA). Chromosomal banding patterns were visualised on a Bio-Rad image analyser following staining with 0.01% (v/v) ethidium bromide. Subsequently, DNA karyotypes at the beginning and end of fermentation could be visually analysed to confirm that the respective yeast inoculums completed the fermentation and that the wine sensory profile could be attributed to the relevant yeast strain.

3.3.3 Small-scale winemaking trials

Cabernet Sauvignon, Merlot, and Shiraz grapes were harvested from vineyards situated on the ARC Nietvoorbij Research farm once the grape sugar in °B titratable acidity (TA) ratio was ± 2.5 as described by Hart et al. (2017a). Subsequently, small-scale (~50 kg) red wines were made in triplicate according to the standard cellar method included in the ARC Infruitec-Nietvoorbij harvest programme 2016 and 2017 (ARC Infruitec-Nietvoorbij wine evaluation committee). Briefly, red grapes were subjected to mechanical destemming where after sulphur dioxide (SO_2) was added at a dosage of 50 mg/L to prevent oxidation. Free-run grape must (juice) was sampled for pH, titratable acidity (TA) and sugar analyses. The juice was allowed one-hour contact with the grape skins for sufficient colour extraction, where after must and skins were pressed at one Bar. Skins were weighed, whilst the volume of the juice was determined. Subsequently, skins and juice were equally aliquoted into the appropriate number of plastic fermentation vessels with a capacity of 50 litres. Two commercial *i.e.* WE372 and MERIT and one experimental *i.e.* ARC Nvbij 6 active dried wine yeasts (ADWY) were re-hydrated separately in sterile distilled H_2O (30 g/300 mL) ~~(Table 1)~~ and inoculated into the grape must at a dosage of 150 mL/50 kg. Thereafter, 50 g/hL diammonium phosphate (DAP) was added to each fermentation vessel. All fermentations were conducted at an ambient temperature of ca. 24 °C and the fermentation “cap” formed by skins were punched-down three times a day to allow carbon-dioxide (CO_2) to escape. Fermentations proceeded until the residual sugar was 50 g/L, where after the fermenting skins and juice were pressed at one Bar. Eighteen litres of juice was siphoned into clean stainless steel fermentation canisters, sealed

with a fermentation lock and were further fermented. Fermenting must samples were taken every 48 hours using food-grade CO_2 to analyse residual glucose/fructose, ethanol, VA, total acidity and pH using an OenoFoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark) as described by Hart et al. (2016). The SO_2 was analysed upon completion of the respective fermentations (residual sugar <2 g/L). The wines were racked off the yeast lees and the total-SO_2 was adjusted to 85 mg/L, followed by cold stabilisation at 0 °C for at least two weeks. Subsequently, wines were filtered and bottled.

3.3.4 Basic chemical analyses of wines using FTIR spectroscopy

Basic chemical parameters of final wines *i.e.* alcohol (%), pH, volatile acidity (g/L), total acidity (g/L) and residual glucose/fructose (g/L) of all red wines were measured using an OenoFoss™ FTIR spectrometer (FOSS Analytical A/S, Denmark).

3.3.5 Gas chromatography (GC) analysis of aroma compounds using a flame ionisation detector (FID)

3.3.5.1 Chemicals used as standards

Yeast synthesised aroma compounds *i.e.* esters, total fatty acids and higher alcohols were quantified by means of calibration standards of the applicable aroma compounds *viz.* ethyl acetate ethyl-3-hydroxybutanoate, ethyl phenylacetate, ethyl propionate, 2-methyl propyl acetate, ethyl decanoate (ethyl caprate), ethyl octanoate, ethyl 2-methylbutyrate, ethyl isovalerate, ethyl hexanoate, ethyl-2-methyl propanoate (ethyl isobutyrate), propionic acid, octanoic acid, valeric acid, acetic acid, hexanoic acid, butyric acid, pentanol, n-propanol, 3-methyl-1-pentanol, 1-octen-3-ol, isoamyl alcohol, acatealdehyde, methanol, acetoin, trans-2-hexenol, cis-3-hexen-1-ol, diethyl succinate, ethyl lactate, hexyl acetate, ethyl butyrate, isovaleric acid, isobutyric acid, butyric acid, butanol, 4-methyl-1-pentanol (internal standard), 3-ethoxyl-1-propanol, 2-phenylethanol and 1-hexanol. All chemical

standards were acquired from various companies *i.e.* Sigma, Germany, Fluka, Switzerland, and Merck, Germany.

3.3.5.2 Extraction and quantification of major metabolites

In the current study, 32 aroma compounds were quantified using a Thermo Scientific TRACE 1300 gas chromatograph (Analytic, City, Switzerland) equipped with an autosampler slipt/splitless injector (GC analytics, Switzerland) coupled to a flame ionisation detector (FID) (Thermo scientific, Italy). These compounds are known as major volatiles (esters, higher alcohols, and fatty acids) contributing to the aroma of wines (Falcao et al., 2008; Robinson et al., 2011).

Briefly 10 mL of the wine sample, 2 mL of diethyl ether (Merck) and 100 µL of the internal standard (4-methyl-2pentanol) diluted to 0.5 mg/L in 12 % v/v ethanol, 2.5 g/L tartaric acid and deionised water at a pH of 3.5 (0.1 M NaOH) was added to a Pyrex glass tube. Extraction was achieved by sonicating the mixture for 30 minutes in an ultrasonic bath (Analab Scientific Instruments Private Limited Vadodara, India) followed by vortexing. The mixture was then centrifuged (Multifuge3S, Kendro Laboratory Products, Germany) for 5 minutes at 4000 rpm. This was followed by the removal of the clear ether layer visible through the Pyrex tube, which was then transferred into a GC vial. These vials were loaded on the GC-FID machine and an autosampler injected the samples into the GC-FID. The conditions for GC-FID were as follows: the initial oven temperature was set at 45 °C, which remained constant for 5 minutes. This was followed by a temperature rise of 3 °C/minute until 100 °C was reached which was stable for 5 minutes and finally raised to 250 °C for 10 minutes. The injector temperature was set at 250 ^{0} C and operated in 5:1 split mode. The aroma compounds were separated with a polar AJ&W 122-3263 DB-FFAP (60 m length × 320 µm internal diameter × 0.5 µm) capillary column (Agilent Technologies, Wilmington, USA). Helium was used as the carrier (1.8 mL/minute) and make up gas (40 mL/minute). Gases used for the FID (hydrogen-air-flame) were air (400 mL/minute) and hydrogen (40 mL/minute).

The ratio of the aroma compounds` peak area and internal standard`s peak area was calculated for the quantification and integration of the compounds. The software used for integration was HP Chemstation software (Rev.B01.03 [204]). The calibration solution contained 12 % v/v ethanol, 2.5 g/L tartaric acid, deionised water at a pH of 3.5 (0.1 M NaOH) known as wine matrix simulate. Varying concentrations (50, 100, 250, 500, 1000, 2500, 5000 µg/L) of the standards solution was added to the wine matrix simulant. The internal standard was also added to this mixture.

3.3.6 Gas chromatography- mass spectrometry (GC-MS) analysis of volatile thiols

3.3.6.1 Chemicals and standards used

Yeast mediated and released volatile thiols *i.e.* 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexyl acetate (3MHA) and 3-mercaptohexan-l-ol (3MH) were quantified by means of calibration standards of the applicable aroma compounds *viz.* 3-mercaptohexan-1-ol(3MH) (Acros Organics, Geel, Belgium), 3-mercaptohexyl acetate (3MHA) (Oxford Chemical, UK), and 4-mercapto-4-methyl pentan- 2-one (Aldrich, Australia), while 4-methoxy-2- methyl-2-mercaptobutane (4M2M2MB) (Acros Organics, Geel, Belgium) was used as internal standard. Hydrochloric acid (Scharlau, Barcelona, Spain), sodium hydroxide pellets (Scharlau, Barcelona, Spain) and sodium sulfate anhydrous powder (Scharlau, Barcelona, Spain), ethyl propiolate (ETP) (Aldrich, CastleHill, NSW, Australia), butylated hydroxyanisole (BHA) (Aldrich, CastleHill, NSW, Australia), and dichloromethane (Merck, Darmstadt, Germany) were used for the extraction of thiols.

3.3.6.2 Extraction and quantification of volatile thiols

The above-mentioned volatiles thiols were extracted from Shiraz, Merlot, and Cabernet Sauvignon wines, respectively (Herbts Johnstone et al. 2013). Briefly for sample preparation 500 µL butylated hydroxyanisole (BHA) (2 mM), 50 µL 4-methoxy-2-methyl-2-mercaptobutane (4M2M2MB) as internal standard and 500 µL ethyl propiolate (ETP) (250 mM) were added to

50 mL red wine. The mixture was then stirred at 500 rpm for five minutes on a magnetic stirrer. Hereafter the pH was adjusted to 10 using NaOH and HCl and mixed again for 10 minutes at 500 rpm. This was followed by centrifugation (Z 366 HERMLEL Labortechnik GmbH, Germany) at 6000 rpm for 10 minutes in falcon tubes. The supernatant was then transferred into a 50 mL beaker whereafter Solid Phase Extraction followed. Cartridges Supelclean™ ENVI-18 (6 mL cartridge volume;1 g sorbent; Supelco, Castle Hill, NSW, Australia), were positioned on a manifold vacuum pump set a 5 kPa and conditioned with 10 mL methanol, followed by 10 mL deionised water. After conditioning a 50 mL wine sample was allowed to flow through the cartridge followed by 5 mL deionised water to wash the cartridge at the same pressure. Cartridges were then left to vacuum dry for 20 minutes at 10 kPa. The thiols retained in the matrix of the cartridges were eluted with 10 mL dichloromethane. Anhydrous sodium sulphate was added to the eluate to get rid of any traces of water, as the presence of water will interfere with the GC analysis. Glass wool was used to filter the eluate to clear it from anhydrous sodium sulphate. The eluate was concentrated under nitrogen gas to a volume of approximately 100 µL and then transfered into GC vials.

Volatile thiols 3-mercaptohexan-1-ol (3MH), 3- mercaptohexyl acetate (3MHA) and 4-mercapto-4-methyl pentan- 2-one were quantified using a Thermo Scientific TRACE 1300 gas chromatograph (Analytic, City, Switzerland) coupled to a Thermo Scientific TSQ 8000 triple quadrupole mass spectrometer detector (MSD). Separation of compounds was performed with a polar Zebron ZB-FFAP (30 m x 0.25 mm x 0.25 µm) (Phenomenex; Torrance, CA, USA) capillary column. The initial oven temperature was set at 60 °C for 1 minute. The initial oven temperature (60 °C for 1 minute) was raised to 100 °C at a rate of 25 °C/min and remained constant for 2 minutes. This temperature was then finally raised to 250 °C at 12 °C/min for 5 minutes. Sample injection was done on the GC injection port maintained at 240 °C operated in splitless mode with the split flow set at 50 mL/minute for 2 minutes. Gas saver was activated for 5 min at 20 mL/minute. Helium at a flow rate of 1.2 mL/minute was used as carrier gas. Both the transfer line and ion source temperatures were set at 250°C. Emission current was set at 75 µA and argon was used as collision gas.

The ratio of the aroma compounds` peak area and internal standard`s peak area was calculated for the quantification and integration of the compounds. The software used for integration was HP Chemstation software (Rev.B01.03 [204]). The calibration solution contained 12 % v/v ethanol, 2.5 g/L tartaric acid, deionised water at a pH of 3.5 (0.1 M NaOH) known as the wine matrix simulate. Varying concentrations of the standards solution (4MMP 10-400 ng/L, 3MHA 50-2000 ng/L, and 3MH 500-20 000 ng/L), was added to the wine matrix simulant. The internal standard was also added to this mixture.

3.3.7 Descriptive sensory evaluation

Red wines were subjected to descriptive sensory evaluation following three months of bottle stabilisation after production by a panel of seven trained wine tasters (judges) as described in Hart et al. (2016). The judges were requested to indicate the intensity of the perceived wine aromas on a sensory evaluation sheet (Appendix I, Fig.1, 2 & 3) consisting of an intensity scale which ranged from undetectable to prominent; or unacceptable to pleasant, respectively. Additionally, judges were required to highlight the most prominent aromas and flavours, amongst others, “blackcurrant”, “black cherry”, “blackberry”, “green pepper”, “herbs” and ”smoky” that they perceived. All wines (approximately 50 mL serving per wine) were served blindly (coded) in a randomised order using standard wine glasses.

3.3.8 Statistical analyses

Chemical and sensory analyses data were recorded and subjected to statistical analysis (Pearson, 1896; 1901; Zou et al*.,* 2006). The data matrix consisted of chemical variables and sensory aroma descriptors. Statistical analyses included a Pearson’s correlation to study the linear relationship between the chemical and sensory variables to standardise the data before performing principal component analyses (PCA) using XLSTAT software (ver. 2015.1.03.15485, Addinsoft).

3.3.9 Proteomic analyses

3.3.9.1 Protein extraction

Fermenting Cabernet Sauvignon, Merlot, and Shiraz grape must (juice) (50 mL) were sampled using food-grade CO_2. Subsequently, 2 mL aliquots were transferred into 2 mL microcentrifuge tubes and yeast cells were harvested by centrifugation at 13000 rpm for three minutes to pellet yeast cells. The aqueous upper layer was discarded and the previous steps were repeated until the yeast pellet weighed 50 mg (0.05 g). The pellets were subjected to protein extraction as previously described by Von den Haar (2007). Briefly, the yeast pellet was liquefied in 400 µL lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2 % (w/v) SDS and 2 % (v/v) 2-mercaptoethanol and sterile deionised water). The cell mixture was incubated in a water bath for 10 minutes at 90 °C to disrupt cells, whereafter 10 µL of 4 M acetic acid was added to the lysates which was incubated for an additional 10 min at 90 °C.

3.3.9.2 Protein quantification (Bradford assays)

The concentration of protein extracts was measured using the Bradford assay (Ernst and Zor, 2010). Samples were prepared by adding 800 µL Bradford reagent, 100 µL deionised water and 100 µL of the protein extract, making up a final volume of 1 mL, in curvets. The absorbance values of this mixture were measured using a mass spectrophotometer at a wavelength of 595 nm, which was extrapolated against a standard curve to determine the protein concentrations of the extracts. A standard curve was drawn up by adding varying amounts of 2 mg/mL BSA (Bovine Serum Albumin) standard (20 mL, 40 mL, 60 mL, 80 mL, 100 mL) to reach varying concentrations (10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, and 50 µg/mL).

3.3.9.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The 1-D SDS PAGE gel is comprised of two parts namely the 5% stacking gel casted over the second gel, the 12% separating gel. The gels were prepared according to Table 1 and left to solidify. A protein ladder (3 µL) was added to the first well, which served as a protein marker (Precision Plus Protein™, Bio-Rad, Madrid, Spain). This was then followed by adding 15 µL (20 µL protein extract and 5 µL loading buffer {2 mL 10 % SDS, 1 mL 20 % glycerol, 1.25 mL 2-mercaptoethanol, 500 µL 1 M Tris-HCl pH 6.8 and 250 µL 1 % bromophenol blue made up to 10 mL deionised water}) of sample in each well. The samples were diluted to a constant concentration of approximately 50 µg/µL as Coomassie Brilliant Blue G-250 (Merck, South Africa) stain`s sensitivity detection limit is 30 ng and samples were heated at 94 °C for 5 minutes before being loaded.

The reservoir of the mini-PROTEAN electrophoresis system (Bio-Rad, Madrid, Spain) was filled with 1× SDS running buffer. The electrophoresis system was first set to run at 100 volts for 15 minutes. Hereafter the volts were increased to 140 and electrophoresed for 1 hour. Upon completion of electrophoresis Coomassie Brilliant Blue G-250 (0.02 % Coomassie blue, 40 % Methanol and 10 % Acetic acid made in distilled water), staining solution was added to the gel and then microwaved for 1 minute to accelerate the stain to infiltrate the proteins. Incubation of the gel was done for 10 minutes. This was followed by a destaining for another 10 minutes using destain solution (10 % methanol and 10 % acetic acid made in distilled water). A Molecular Imager PharosFX Plus System (Bio-Rad, Madrid, Spain) was used to visualise gel images as described by Ngara et al. (2008).

Table 1. Chemicals used to prepare SDS PAGE gels

12 % Separating gel	**Volumes (µL)**	**5% Stacking gel**	**Volumes (µL)**
Deionised water	2150	Deionised water	1500
40 % Bis-Acrylamide (BioRad)	1500	40 % Bis-Acrylamide	315
1.5 Tris (pH 8.8) (Merck)	1250	0.5 Tris (pH 6.8)	625
10 % SDS (Merck)	50	10 % SDS	25
10 % *APS (Merck)	50	10 % APS	25
*TEMED (Sigma)	5	TEMED	2,5

*APS (ammonium sulphate)

*TEMED (Tetramethylethylenediamine)

3.4 RESULTS AND DISCUSSION

3.4.1 Pulsed-field gel electrophoresis (PFGE)/Contour clamped homogeneous electric field (CHEF) DNA karyotyping

For the contour-clamped homogeneous electric field (CHEF) analysis, yeast strains were isolated at the end of fermentation and compared to the starter cultures (WE372, MERIT, and ARC Nvbij 6). Contour-clamped homogeneous electric field (CHEF) DNA karyotypes of commercial reference yeasts *i.e.* WE372 and MERIT and the experimental *i.e.* ARC Nvbij 6 starter cultures (inoculums), matched that of relevant cultures isolated at the end of Shiraz (Fig. 1a), Merlot (Fig. 1b) and Cabernet Sauvignon (Fig. 1c) fermentations, respectively. This implies that the inoculated yeast propagated sufficiently during the fermentation and were sufficient to eradicate a substantial amount of wild non-*Saccharomyces* and *Saccharomyces* yeast during the progression of the fermentation. Therefore, variation in final wine chemical, sensory and metabolite levels can be attributed to the inoculated yeast strain, as it was the only variable in this study.

3.4.2 Small-scale winemaking trials

Small-scale winemaking was conducted by using three different *Saccharomyces cerevisiae* strains *i.e.* a naturally isolated experimental yeast strain ARC Nvbij 6, and two commercial strains *i.e.* MERIT and WE372 .The chemical composition of the base grape must (juice) used for the fermentations during both the 2016 and 2017 vintages are summarised in Tables 2, 3, and 4. The grape must chemical composition showed negligible variation between grape cultivars used *i.e.* Cabernet Sauvignon, Merlot, and Shiraz as well the two vintages. It was also observed that the grape must pH ranged from 3.56 to 4.11, which is higher than the sought-after pH 3.5, as resultant wines can potentially lack varietal aroma and flavours (Koegelenberg, 2003; Belloch et al., 2008). Furthermore, sugar (°Balling) of all cultivars were in alignment to what is used in commercial winemaking (C. Paulsen, Personal communication, 2017). Total sulphur dioxide (SO_2) in all grape juice were adjusted to 50 mg/L according to the

ARC Nietvoorbij harvest programme, as total SO_2 were below the recommended level. Wine total acids (TA) were also in accordance to that of commercial winemaking.

All yeast strains completed the fermentation within five, seven and eight days following inoculation, during the fermentation of 2016 Shiraz, Merlot and Cabernet Sauvignon grape must, respectively (Fig. 2a, b & c). However, all yeast strains completed the fermentation within nine days following inoculation during 2017 in three cultivars mentioned-above (Fig. 3a, b and c). This observation highlights the influence of vintage on the winemaking process; hence, trials are conducted over more than one vintage to address this aspect. Nonetheless, no noticeable differences within the same cultivar in terms of fermentation rate was observed amongst different yeast stains. The exponential growth phase commenced on day two following inoculation of all yeast strains and in all cultivars during both vintages. The experimental yeast ARC Nvbij 6 appeared to consume the sugar at a slightly faster rate than the commercial reference yeasts. This phenomenon was more apparent during the 2017 Shiraz and Cabernet Sauvignon wine production.

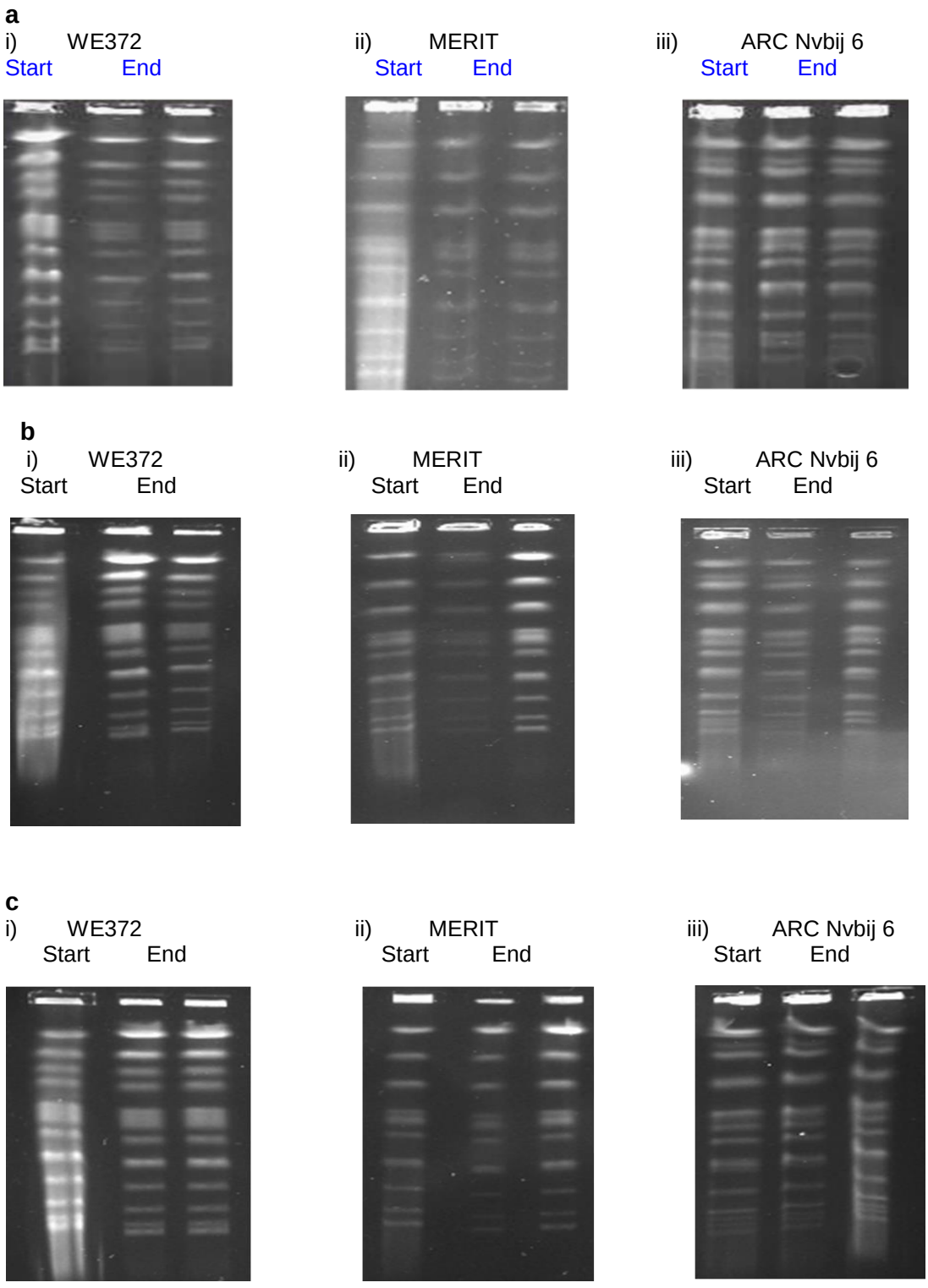

Figure 1. Contour clamped homogeneous electric field (CHEF) DNA karyotypes of commercial *i.e.* i) WE372 and ii) MERIT and one experimental *i.e.* iii) ARC Nvbij 6 red wine yeasts (conserved in the ARC Infruitec-Nietvoorbij microbial culture collection (ARC Inf CC) that was used to produce **a)** Shiraz, **b)** Merlot and **c)** Cabernet Sauvignon wines, respectively.

*Start= Yeast starter culture; End = Yeast colonies randomly isolated at end of fermentation.

Table 2. Analyses of Shiraz grape must used for small-scale fermentations[1].

Vintages (Harvest)	Sugar (°B)	pH	Total acids (g/L)	Total SO_2(mg/L)
2016	26.2	3.70	4.3	**8**
2017	**26.8**	**3.73**	**3.19**	**<2**

[1]Average values of triplicate analyses. Must analyses done by OenoFoss™ (Microbiology, Post-Harvest and Wine Technology, ARC Infruitec-Nietvoorbij, Stellenbosch).

Table 3. Analyses of Merlot grape must used for small-scale fermentations[1].

Vintages (Harvest)	Sugar (°B)	pH	Total acids (g/L)	Total SO_2 (mg/L)
2016	24.4	3.94	3.32	**<2**
2017	**26.6**	**3.61**	**3.21**	**2**

[1]Average values of triplicate analyses. Must analyses done by OenoFoss™ (Microbiology, Post-Harvest and Wine Technology, ARC Infruitec-Nietvoorbij, Stellenbosch).

Table 4. Analyses of Cabernet Sauvignon grape must used for small-scale fermentations[1].

Vintages (Harvest)	Sugar (°B)	pH	Total acids (g/L)	Total SO_2 (mg/L)
2016	26.2	4.11	4.27	**2**
2017	**25.4**	**3.56**	**3.45**	**<2**

[1]Average values of triplicate analyses. Must analyses done by Oenofoss™ (Microbiology, Post-Harvest and Wine Technology, ARC Infruitec-Nietvoorbij, Stellenbosch).

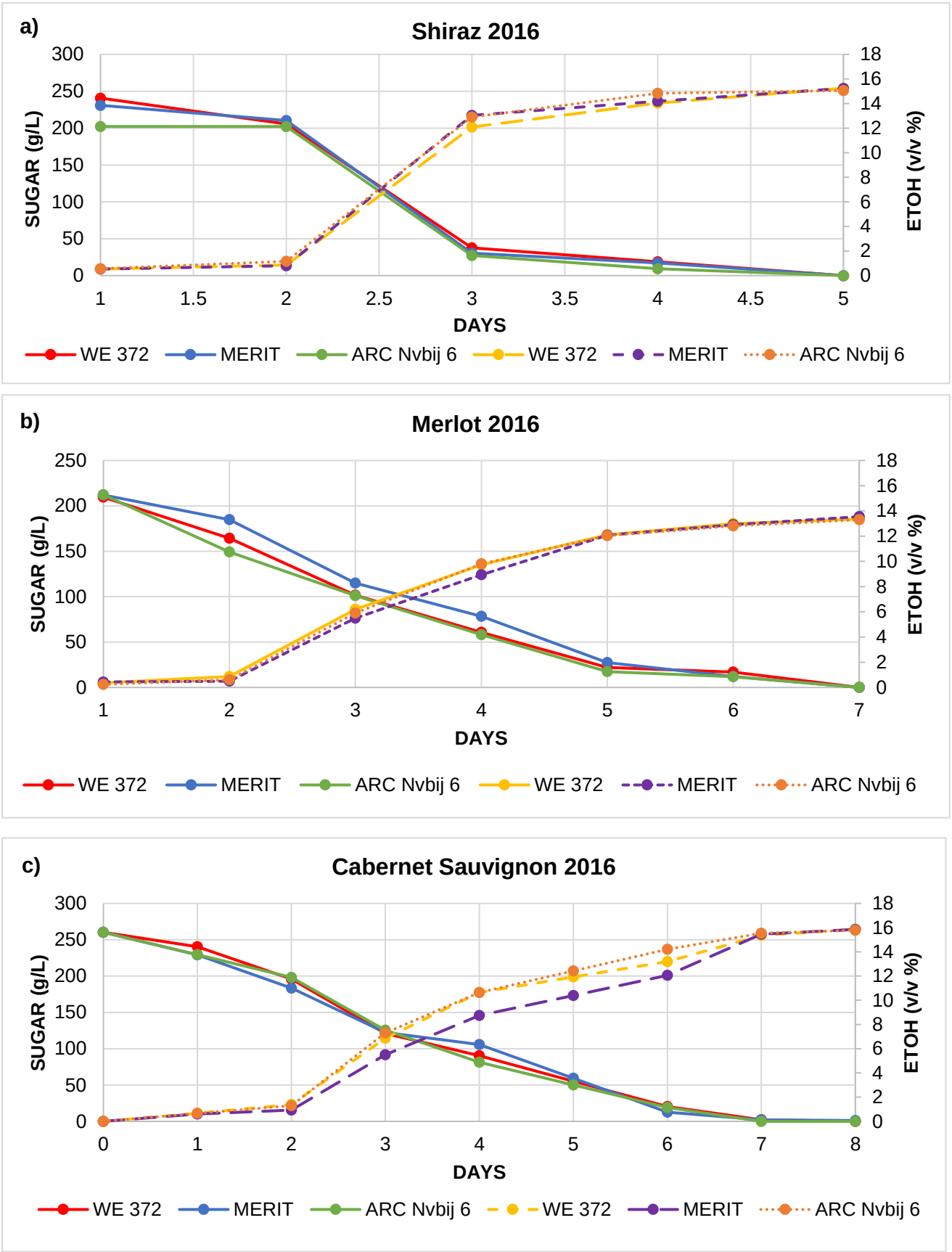

Figure 2. Sugar utilization (solid lines) and alcohol accumulation (perforated lines) during the 2016 vintage of WE372, MERIT, and ARC Nvbij 6 used to ferment a) Shiraz, b) Merlot, and c) Cabernet Sauvignon. Fermentation was monitored throughout alcoholic fermentation using OenoFoss™.

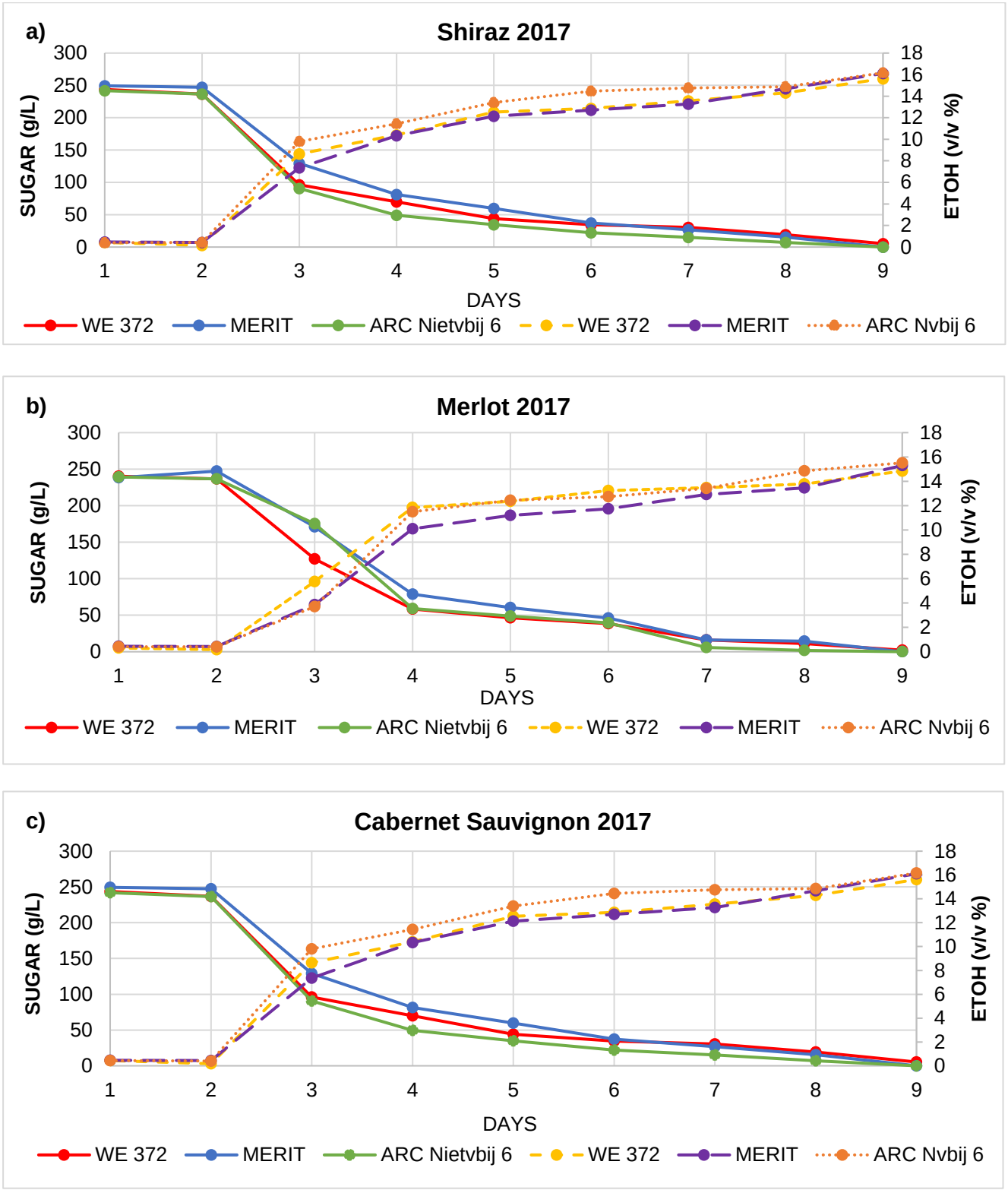

Figure 3. Sugar utilization (solid lines) and alcohol accumulation (perforated lines) during the 2017 vintage of WE372, MERIT, and ARC Nvbij 6 used to ferment a) Shiraz, b) Merlot, and c) Cabernet Sauvignon. Fermentation was monitored throughout alcoholic fermentation using OenoFoss™.

3.4.3 Basic chemical analyses of wines using FTIR spectroscopy

Data of basic chemical parameters of Shiraz, Merlot, and Cabernet Sauvignon wines produced with commercial reference yeasts *i.e.* MERIT, WE372 and experimental yeast ARC Nvbij 6 following OenoFoss™ FTIR analyses are shown as principle component analysis (PCA) biplots. The Shiraz (Fig. 4a & Fig. 5a), Merlot (Fig. 4b & 5b), and Cabernet Sauvignon (Fig. 4c & 5c) wines produced by the experimental strains *i.e.* ARC Nvbij 6 and MERIT had a negative association with VA across both vintages. Shiraz wines produced with the WE372 also had a negative association with VA across both vintages (2016 and 2017). Contrasting observations were made for WE372 produced 2016 Merlot and 2016 and 2017 Cabernet Sauvignon wines as these wines had a positive association with VA. Volatile acidity is a major problem in the wine industry as VA gives wines an unpleasant vinegar-like aroma and flavour thus methods to alleviate this issue are sought-after (Vilela et al., 2013). Volatile acidity can adversely affect the quality of the wine, with acetic acid being the primary compound responsible (Vilela-Moura et al., 2010; Hart et al., 2016). The undesirable vinegar-like aroma can be perceived at acetic acid concentrations as low as 0.8 g/L in the wines (Vilela et al., 2013). This is lower than the legal limit (1.2 g/L) of acetic acid permitted in wines (OIV, 2012; Sirén et al., 2015). Acetic acid can be synthesised by wine yeast during winemaking (Cordente et al., 2013; Luo et al., 2013). Thus, wine yeast strains, such as the experimental yeast ARC Ntvbij 6 investigated in the current study, producing very low levels of acetic acid will be an asset to the wine industry. All chemical parameters, especially VA of all wines were within legal limits (Du Toit, 2001).

Nearly all wines produced with the commercial yeast MERIT had a positive association with pH with the exception of 2016 Shiraz wines. Nonetheless, all the yeast strains used in the current study produced wines with pH values ranging between 3.66 to 4.55 (data not shown). Noteworthy, 2016 Cabernet Sauvignon wines had fairly high pH values, *i.e.* between 4.16 to 4.55 (data not shown). This is due to the high pH value of the Cabernet Sauvignon grape must used to produce the wines (Pambianchi, 2001). Grape juice with pH values exceeding 4 is commonly reported in environments with high temperatures (Sigler and Freiburg, 2008). It is

assumed that the Cabernet Sauvignon grapes may have had more sun exposure compared to Merlot and Shiraz during the ripening period prior to the 2016 harvest. The microbiological stability of the wines was however not affected by this factor. Vahl et al. (2013) stated that that high pH values have the disadvantage of impairing the microbiological stability of wine. Cabernet Sauvignon wines from the 2016 harvest displayed no fruity flavour aroma and flavour and this can possibly be attributed to the high pH values. Hart et al. (2016) reported that wines within a certain pH range *i.e.* 3.3 for white and 3.8 for red (Pambianchi, 2001) are perceived to be fruitier. Futhermore, WE372 produced wines, had a positive association with total acidity, which is closely related to volatile acidity. All Shiraz, Merlot, and Cabernet Sauvignon wines produced with the experimental yeast ARC Nvbij 6, irrespective of the vintage had a positive association with alcohol (Fig. 4a, b, c, & Fig. 5a, b, & c). However, no noticeable difference was observed in the alcohol content of the wines produced during the 2016 harvest season. The same trend was observed during the 2017 harvest, with the exception of WE372, which noticably differed from MERIT and the experimental yeast ARC Nvbij 6 in terms of alcohol content. Baker and Ross (2014) reported that wines with higher alcohol values positively contributed to the perceived sensory profile of red wines, as wines with higher alcohol values tend to have a ‘longer finish’ or aftertaste. This is an important attribute of wine quality and is defined as the lingering taste and aroma after swallowing (Baker and Ross, 2014).

3.4.4 Descriptive sensory evaluation

The principle component analysis (PCA) biplot of descriptive sensory evaluation shows that both commercial reference yeasts *i.e.* MERIT and WE372 and the experimental yeast strain *i.e.* ARC Nvbij 6), produced 2016 Shiraz wines with a positive association with ‘jammy’, ‘smoky’ and ‘spicy’ flavours and aromas (Fig. 4d). These flavours and aromas are associated with a typical Shiraz wine (Goldstein and Goldstein, 2006). The same trend was observed during the 2017 harvest season (vintage) with the exception of the MERIT produced Shiraz wines (Fig. 5d). Rotundone, the compound responsible for the ‘peppery’, spicy’, and ‘herbs’ aroma

in Shiraz wines (Wood et al., 2008; Caputi et al., 2011) could have been present in concentrations above its sensory detection threshold, thus masking the 'red fruit' flavour and aromas also associated with Shiraz wines. The wines could unfortunately not be analysed for this compound as no accredited local laboratory does the analysis. The sensory panel also indicated that commercial yeast strain, WE372 produced 2017 Shiraz wines with 'berry' aromas. It could be that WE372 produced 2017 Shiraz wines with higher concentrations of 'fruit'; berry' (also referred to as red and black fruit) enhancing metabolites. This is in agreement with the GC-FID results, where the WE372 produced wines had the highest overall ester value compared to the wines produced from the other two yeast strains (Tables 6 & 7; Appendix II Fig. 4a & 5a). The MERIT produced 2017 Shiraz wines, on the other hand, were perceived to be 'vegetative' (Fig. 5d). This could be that lower temperatures were experienced during 2017, which are known to enhance 'vegetative; 'herbaceous' and 'peppery' flavours and aromas due to grape-derived aroma compounds *i.e.* methoxypyrazines which are sensitive to high temperatures (De Klerk, 2007), thereby masking the 'fruity and 'floral' aroma enhancing metabolites produced normally associated with warmer viticultural areas.

Merlot wines produced with MERIT during the 2016 vintage had a positive association with 'floral' and 'violet' flavours and aromas, which are varietal aromas, whilst that produced with WE372 had a positive association with 'fruity' and 'berry' with hints of 'spicy' flavours and aromas (Fig. 4e). The yeast strain ARC Nvbij 6 produced 2016 Merlot wines with a positive association with floral/violet flavours and aromas, however to a lesser extent than the MERIT produced wines. All aromas mentioned above are regarded as typical Merlot flavours and aromas. This observation is a clear indication that the wine yeast starter culture (inoculum) can modulate wine varietal aromas and flavours (Du Plessis et al., 2017; Hart et al., 2017a). Furthermore, Swiegers et al. (2009) and (Von Mollendorff, 2013) also reported on the effect of wine yeast to modulate varietal aromas of white wine, especially Sauvignon blanc. Merlot wines produced with MERIT during the 2017 vintage, however, had a positive association with 'spicy' flavours and aromas, whilst that produced with WE372 had a positive association with 'floral', 'violet', 'fruity' and 'berry' flavours and aromas (Fig. 5e). In contrast, the yeast ARC Nvbij 6 produced 2017 Merlot wines with a negative association with floral', 'violet', 'fruity' and

'berry', however, the wines had a stronger association with 'spicy' nuances than wines produced with WE372 (Fig. 5e). Overall, the commercial yeast strain, WE372 produced 2017 Merlot wines were consistent compared to that of 2016, as these wines were perceived to be 'fruity' during both vintages (Fig. 4e & 5e). Both MERIT and ARC Nvbij 6 strains produced Merlot wines with differing association with 'spicy' and 'floral' aromas during the two vintages (Fig. 4e & 5e). Nonetheless, all aromas mentioned above are associated with a typical Merlot flavour and aroma. This observation is a clear indication that besides the wine yeast starter culture (inoculum) observed during 2016, vintage can also have an effect on wine varietal aromas and flavours (Louw et al., 2010).

Cabernet Sauvignon wines produced with WE372 and ARC Nvbij 6 during the 2016 vintage had a positive association with 'colour intensity' and 'overall quality', whilst MERIT produced wines had an increased positive association with the 'vegetative fresh' (*e.g.* green apple) aroma (Fig. 4f). It is noteworthy, that none of the yeast strains produced a 2016 Cabernet Sauvignon with a strong positive association with 'spicy' and 'berry' aromas and flavours, both of which were included as sensory descriptors for all three cultivars. This observation is a clear indication that besides the wine yeast starter culture (inoculum) and vintage, the cultivar can also have an effect on final wine aromas and flavours (Louw et al., 2010). Nonetheless, as was observed during both 2016 and 2017 vintages as well as Shiraz and Merlot wines, all yeast strains managed to produce wines with varietal aromas and flavours, albeit to varying degrees. Descriptive sensory evaluation of the 2017 Cabernet Sauvignon wines again complements this observation made during 2016, as only MERIT produced wines with a positive association with 'vegetative fresh' (*e.g.* green apple, grass etc.) and 'vegetative dry' (*e.g.* hay etc.) aromas (Fig. 5f). The yeast WE372 produced Cabernet Sauvignon wines were perceived to have 'red fruit' (berry) and 'spicy' nuances, whilst the ARC Nvbij 6 strain produced 2017 Cabernet Sauvignon had stronger associations with less desirable 'vegetative cooked' (*e.g.* asparagus, beans) aromas and 'overall quality' (Fig. 5f). Nonetheless, ARC Nvbij 6 proved that it has a role to play in the production of varietal red wines based on the chemical and sensory attributes of Shiraz, Merlot, and Cabernet Sauvignon wines produced.

Different wine yeast metabolises grape juice differentially and thus produce wines with distinct sensory profiles (Pretorius, 2000; King et al. 2008). This is particularly important to the wine makers as a specific yeast strain can be used to modulate a specific wine style, giving the wine makers more options. The wine industry is a very competitive and congested market redering wine yeast that can produce wines with distinct flavours and aromas highly sought after (Bellon et al., 2011). Furthermore, it can be said that distinctness is equivalent to prestigious wines in a competitive market such as the wine industry, hence wine yeast metabolite production and release was investigated in this study.

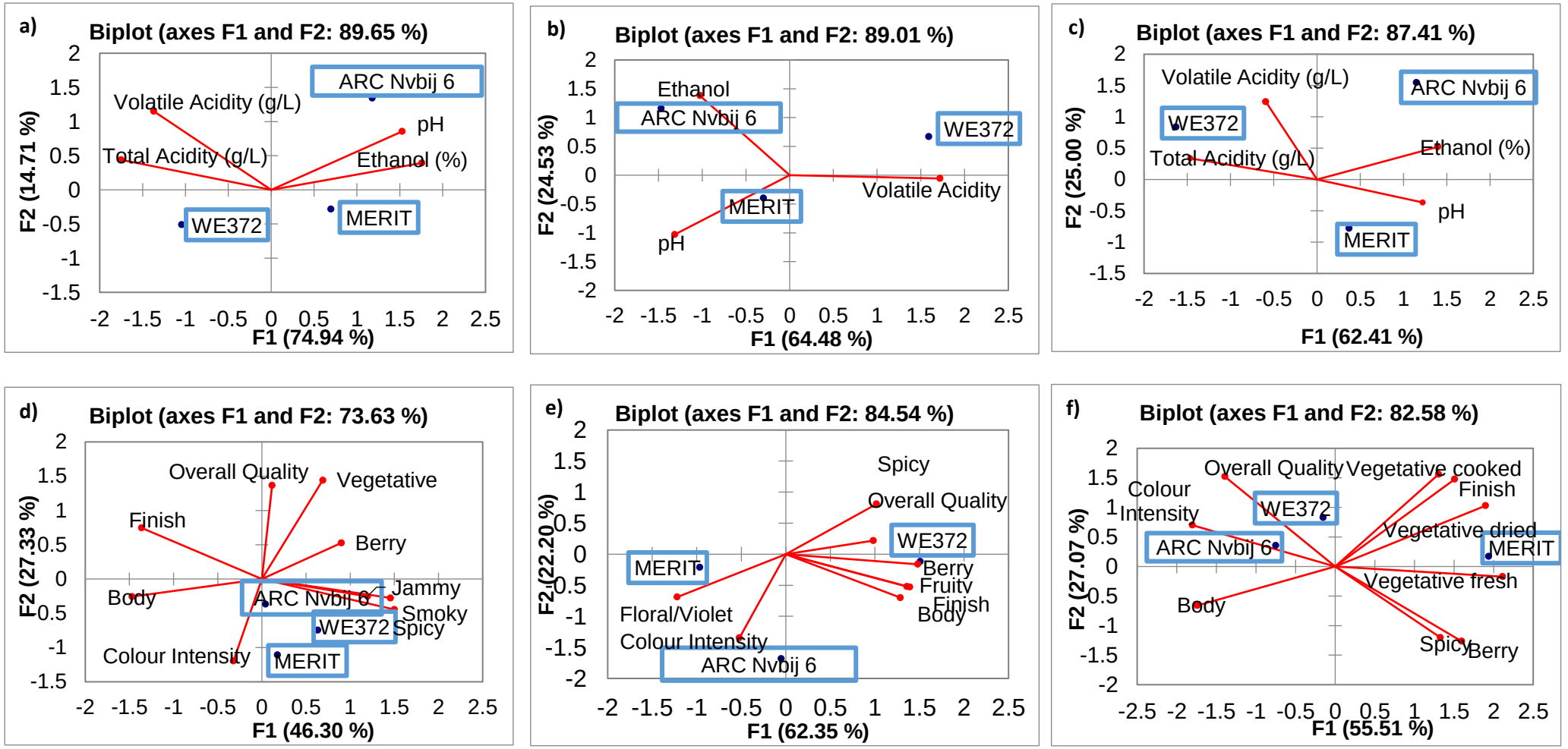

Figure 4. PCA biplots of basic chemical parameters of small-scale 2016 **a)** Shiraz, **b)** Merlot and **c)** Cabernet Sauvignon wine and descriptive sensory analysis of small-scale 2016 **d)** Shiraz, **e)** Merlot and **f)** Cabernet Sauvignon following fermentation by commercial red wine yeasts *i.e.* WE372 and MERIT and the naturally isolated experimental yeast strain *i.e.* ARC Nvbij 6. Average values of triplicate fermentations.

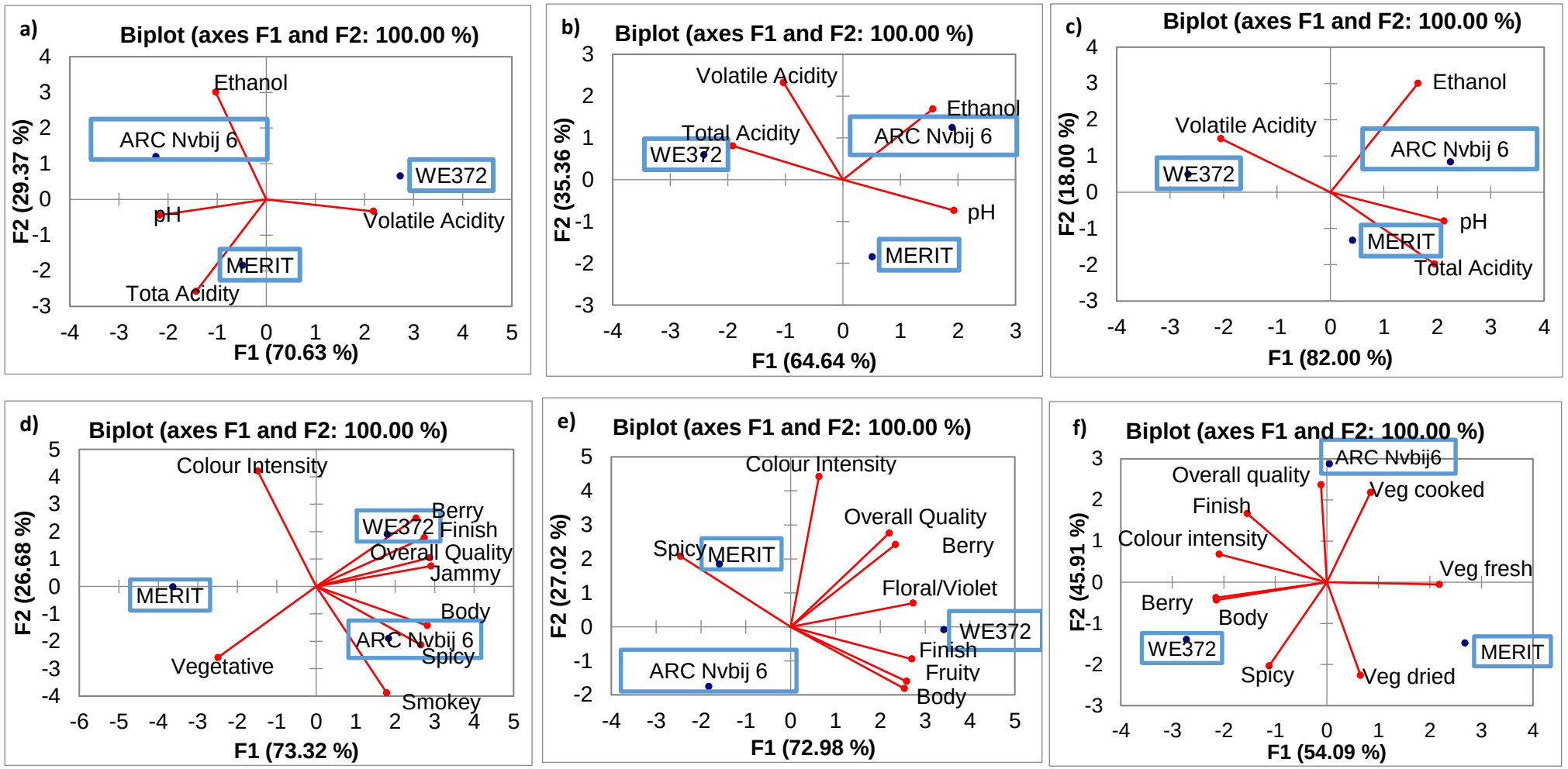

Figure 5. PCA biplots of basic chemical parameters of small-scale 2017 **a)** Shiraz, **b)** Merlot and **c)** Cabernet Sauvignon wine and descriptive sensory analysis of small-scale 2017 **d)** Shiraz, **e)** Merlot and **f)** Cabernet Sauvignon following fermentation by commercial red wine yeasts *i.e.* WE372 and MERIT and the naturally isolated experimental yeast strain *i.e.* ARC Nvbij 6. Average values of triplicate fermentations.

3.4.5 Aroma compound analyses using GC-FID

Major wine volatile aroma compounds, namely esters (imparts fruit and berry aromas), higher alcohols (of which some enhances wine complexity) and fatty acids (contributes to wine acidity in moderate concentrations) in Shiraz, Merlot, and Cabernet Sauvignon wines produced using the commercial reference yeasts *i.e.* MERIT and WE372, and the experimental yeast *i.e.* ARC Nvbij 6 were measured using a GC-FID upon completion of fermentation. A total of 32 aroma compounds were identified in all three cultivars which are summarised in Tables 5-10.

The GC-FID analyses showed that WE372 (40.49 ± 6.22 mg/L) produced 2016 Shiraz wines with noticeably more ethyl acetate, the main ester compound associated with 'apple', 'pineapple', and 'fruity' aromas, than that produced by MERIT (32.59 ± 2.12 mg/L) and ARC Nvbij 6 (32.20 ± 4.09 mg/L), respectively (Table 5). The ethyl acetate concentration also exceeded its olfaction perception threshold (Rossouw, 2009; Lapalus 2016), which correlates with the sensory data (Fig. 4d) as all the 2016 Shiraz wines were perceived to be fruity (jammy). However, excessive levels of ethyl acetate are associated with wine off-odour, namely nail polish remover (Mateos et al., 2006). It is noteworthy that, higher ethyl acetate levels were also reported to accentuate VA, as it is the second highest contributor thereof after acetic acid.

The yeast ARC Nvbij 6 (270 ± 22.03 mg/L) produced 2016 Shiraz wines with noticeably less acetic acid, the main volatile acid (imparts unpleasant vinegar off-odours), than that produced by both WE372 (295 ± 11.72 mg/L) and MERIT (364 ± 87.52 mg/L), respectively (Table 5). Additionally, ARC Nvbij 6 (207.18 ± 22.21 mg/L) produced 2016 Shiraz wines with noticeably less isoamyl alcohol (imparts unpleasant solvent off-odours) than that produced by WE372 (284 ± 35.59 mg/L) and MERIT (251.03 ± 23.81 mg/L), respectively (Table 5). The WE372 produced 2016 Shiraz wines had the highest concentration of diethyl succinate (1.19 ± 0.19 mg/L), that also exceeded its aroma threshold (Rossouw, 2009; Lapalus 2016). This observation complements the sensory data as WE372 produced wines were perceived to be 'fruity'; 'jammy' and 'berry' (Fig. 4d). Although diethyl succinate in ARC Nvbij 6 (0.70 mg/L ± 0.11 mg/L) produced wines also exceeded the aroma threshold, wines were not perceived to be fruity (Fig. 5d). However, the

wines had a strong association with ‘smoky’ and ‘spicy’ aromas that is usually associated with a typical varietal Shiraz wine. The aroma compound *i.e.* rotundone that naturally occurs in Shiraz grapes, which happen to impart ‘peppery’ and ‘spicy’ aromas, could have been responsible for this observation as mentioned previously. The yeast MERIT produced 2016 Shiraz wines had the highest concentration of ethyl-3-hydroxybutanoate, which imparts ‘red berry’ aromas (Pineau et al., 2009). However, the concentrations did not exceed the sensory threshold; hence, sensory evaluation revealed that wines had a weak association with berry aromas (Fig. 4d). It was also observed that both commercial references WE372 and MERIT produced 2016 Shiraz wines with noticeably more 2-phenyl ethanol (imparts honey-like and rose aromas) (Musarurwa et al., 2016) of the straight chain higher alcohols, than that produced with ARC Nvbij 6 (Table 5). Fermenting yeast strains synthesise these higher alcohols during alcoholic fermentation, hence the yeast strain is an important contributor to modulate or enhance wine aroma and flavour (Lambrechts and Pretorius, 2000). Overall, the yeast ARC Nvbij 6 produced noticeably less of the unwanted compounds. This is a positive observation, as excessive levels of aforementioned metabolites will have a negative effect on wine sensory quality.

Table 5. Major wine volatile aroma compounds, namely esters, higher alcohols and fatty acids measured using gas chromatography of small-scale 2016 Shiraz wines produced at the Nietvoorbij research cellar following fermentation with a natural experimental yeast strain ARC Nvbij 6 and commercial yeast strains MERIT and WE372. Average values of triplicate fermentations.

Aroma compounds (mg/L)	WE372			MERIT			ARC Nvbij 6		
2-Phenyl_Ethanol	46.14	±	3.08	38.75	±	3.61	32.84	±	5.27
2-Phenylethyl_Acetate	0.73	±	0.09	0.55	±	0.06	0.54	±	0.06
3-ethoxy-1-propanol	2.17	±	0.30	2.73	±	1.23	2.19	±	0.14
3-methyl-1-pentanol	2.56	±	0.06	1.16	±	0.02	1.47	±	0.60
4-methyl-1-pentanol	0.33	±	0.08	0.18	±	0.13	0.32	±	0.08
Acetic_Acid	295.95	±	11.72	364.09	±	87.52	270.46	±	22.03
Acetoin	0.45	±	0.04	0.28	±	0.04	0.33	±	0.05
Butanol	4.33	±	0.40	1.95	±	0.72	2.84	±	0.66
Butyric_Acid	0.23	±	0.09	0.12	±	0.06	0.17	±	0.06
Diethyl_Succinate	1.19	±	0.19	1.04	±	0.14	0.70	±	0.11
Ethyl_acetate	*40.49	±	6.22	32.59	±	2.12	32.20	±	4.09
Ethyl_butyrate	0.29	±	0.03	0.17	±	0.03	0.22	±	0.02
Ethyl_Caprate	0.13	±	0.02	0.07	±	0.01	0.09	±	0.01
Ethyl_Caprylate	0.19	±	0.03	0.14	±	0.03	0.17	±	0.03
Ethyl_Hexanoate	0.02	±	0.03	0.00	±	0.00	0.00	±	0.00
Ethyl_Lactate	12.74	±	0.30	5.76	±	0.12	7.29	±	3.00
Ethyl_phenylacetate	2.24	±	0.07	1.91	±	0.30	1.87	±	0.21
Ethyl-3-hydroxybutanoate	0.63	±	0.09	0.36	±	0.01	0.37	±	0.11
Hexanoic_Acid	7.47	±	7.19	5.47	±	5.75	11.62	±	4.75
Hexanol	2.97	±	0.38	2.86	±	0.42	2.68	±	0.37
Hexyl_Acetate	0.21	±	0.05	0.15	±	0.00	0.18	±	0.02
Isoamyl_Acetate	4.59	±	0.97	2.90	±	0.32	3.04	±	0.92
Isoamyl_alcohol	284.55	±	35.59	251.03	±	23.81	207.18	±	22.21
Isobutanol	34.12	±	3.74	51.63	±	12.99	30.69	±	2.26
Isobutyl-Acetate	0.07	±	0.02	0.10	±	0.02	0.05	±	0.04
Isobutyric_acid	1.77	±	0.16	1.72	±	0.47	1.10	±	0.26
Iso-Valeric_Acid	1.88	±	0.17	1.55	±	0.30	1.37	±	0.16
n-propanol	90.18	±	10.81	33.52	±	26.88	77.41	±	5.52
Octanoic_Acid	1.37	±	0.11	1.12	±	0.14	1.20	±	0.13
Pentanol	0.30	±	0.01	0.19	±	0.13	0.29	±	0.02
Propionic_Acid	2.37	±	0.8	1.12	±	0.42	1.84	±	0.28
Valeric_Acid	0.93	±	0.05	0.68	±	0.03	0.83	±	0.04

The GC-FID analyses showed that MERIT (49.32 ± 2.33 mg/L) produced 2017 Shiraz wines with marginally more ethyl acetate, the main ester compound associated with 'fruity' aromas, than that produced by ARC Nvbij 6 (48.60 ± 12.78 mg/L) and WE372 (47.64 ± 2.64 mg/L), respectively (Table 6). However, MERIT produced wines with a negative association with 'red fruit' aromas, whilst that of the other two strains had a positive association with, amongst others, red fruit aroma (Fig. 4d). Both commercial references MERIT (2.42 ± 0.31 mg/L) and WE372 (2.40 ± 0.21 mg/L) produced wines with isoamyl acetate levels above their sensory detection threshold (Rossouw, 2009), as well as the experimental yeast ARC Nvbij 6 (2.18 ± 0.87 mg/L).

In terms of acetic acid, the main contributor to wine total fatty acids and vinegar-like off-flavour, ARC Nvbij 6 (249.04 ± 96.50 mg/L) produced 2017 Shiraz wines with noticeably lower levels than wines produced by WE372 (255.29 ± 18.80 mg/L) (Table 6). This observation complements the FTIR analyses, as the ARC Nvbij 6 produced wines also had a negative association with volatile acidity (Fig. 5a). Additionally, ARC Nvbij 6 (291.33 ± 23.20 mg/L) produced 2017 Shiraz wines with noticeably less isoamyl alcohol (imparts unpleasant solvent off-odours) than wines produced by WE372 (332.73 ± 30.56 mg/L) and MERIT (422.23 ± 55.97 mg/L), respectively (Table 6).

The yeast MERIT (0.56 ± 0.04 mg/L) produced 2017 Shiraz wines with noticeably more 2-phenylethyl acetate, which imparts 'fruity' aromas than wines produced by ARC Nvbij 6 (0.51 ± 0.20 mg/L) and WE372 (0.49 ± 0.02 mg/L), respectively (Table 6). All yeasts produced this aroma compound above its sensory detection threshold (0.25 mg/L). Furthermore, the WE372 (7.46 ± 0.09 mg/L) produced 2017 Shiraz wines had the highest concentration of diethyl succinate (imparts 'fruity' aroma), than wines produced by MERIT (6.91 ± 0.33 mg/L) and ARC Nvbij 6 (5.71 ± 3.51 mg/L), respectively. All yeasts did however produce this aroma compound above its sensory detection threshold (1.20 mg/L). It is noteworthy, that only WE372 produced wines with a positive association with red fruit (berry) aroma based on descriptive sensory analyses, whilst ARC Nvbij 6 produced wines with a strong association with 'smoky' and 'spicy' aromas that is usually associated with a typical varietal Shiraz wine. The same observation was made in the 2016 Shiraz wines.

The yeast MERIT (0.60 ± 0.03 mg/L) produced 2017 Shiraz wines with higher levels of ethyl-3-hydroxybutanoate (imparts 'red berry' aromas) (Pineau et al., 2009) than wines produced by WE372 (0.55 ± 0.02 mg/L) and ARC Nvbij 6 (0.45 ± 0.08 mg/L), respectively (Table 6). However, wines produced by the former had a negative association with berry aromas (Fig. 5d). It was also observed that the commercial reference WE372 (46.86 ± 3.61 mg/L) produced 2017 Shiraz wines with noticeably more 2-phenyl ethanol (imparts honey-like and rose aromas) (Musarurwa et al., 2016), than wines produced with MERIT (30.64 ± 2.31 mg/L) and ARC Nvbij 6 (18.93 ± 1.08 mg/L), respectively. This observation was complemented by descriptive sensory analyses as WE372 produced wines with a positive association with 'jammy' aroma, which has a sweet connotation as it the case with honey (Fig. 5d). Nonetheless, the experimental yeast still produced wines with typical Shiraz aroma nuances. Overall, the yeast ARC Nvbij 6 consistently (both vintages) produced varietal aromatic Shiraz wines with noticeably less of the unwanted compounds that are known to be detrimental to wine organoleptic quality. This is a positive observation, as the vintage did not affect the experimental yeast strains performance.

Table 6. Major wine volatile aroma compounds, namely esters, higher alcohols and fatty acids measured using gas chromatography of small-scale 2017 Shiraz wines produced at the Nietvoorbij research cellar following fermentation with a natural experimental yeast strain ARC Nvbij 6 and commercial yeast strains MERIT and WE372. Average values of triplicate fermentations.

Aroma compounds (mg/L)	WE372			MERIT			ARC Nvbij 6		
2-Phenyl_Ethanol	46.86	±	3.61	30.64	±	2.31	18.93	±	1.08
2-Phenylethyl_Acetate	0.49	±	0.02	0.56	±	0.04	0.51	±	0.20
3-ethoxy-1-propanol	8.01	±	0.15	1.60	±	0.02	6.34	±	0.95
3-methyl-1-pentanol	6.74	±	0.28	5.46	±	0.37	4.99	±	1.46
4-methyl-1-pentanol	0.31	±	0.07	0.23	±	0.01	0.26	±	0.03
Acetic_Acid	357.48	±	6.88	412.66	±	8.79	285.22	±	40.60
Acetoin	0.45	±	0.03	0.25	±	0.01	0.38	±	0.17
Butanol	3.44	±	0.07	1.22	±	0.10	2.55	±	0.98
Butyric_Acid	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Diethyl_Succinate	7.46	±	0.09	6.91	±	0.33	5.71	±	3.51
Ethyl_Acetate	47.64	±	2.64	49.32	±	2.33	48.60	±	12.78
Ethyl_butyrate	0.22	±	0.01	0.21	±	0.03	0.23	±	0.08
Ethyl_Caprate	0.08	±	0.00	0.08	±	0.01	0.07	±	0.03
Ethyl_Caprylate	0.13	±	0.01	0.15	±	0.01	0.15	±	0.07
Ethyl_Hexanoate	0.00	±	0.00	0.00	±	0.00	0.03	±	0.04
Ethyl_Lactate	33.55	±	1.41	27.19	±	1.84	24.82	±	7.27
ethyl_phenylacetate	0.72	±	0.01	0.78	±	0.01	0.75	±	0.02
Ethyl-3-hydroxybutanoate	0.55	±	0.02	0.60	±	0.03	0.45	±	0.08
Hexanoic_Acid	0.00	±	0.00	0.69	±	0.98	3.53	±	2.63
Hexanol	2.52	±	0.23	2.54	±	0.16	3.26	±	1.13
Hexyl_Acetate	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Isoamyl_Acetate	2.42	±	0.31	2.40	±	0.21	2.18	±	0.87
Isoamyl_alcohol	255.29	±	18.80	247.36	±	15.93	249.04	±	96.50
Isobutanol	37.78	±	4.42	65.97	±	1.58	41.63	±	9.16
Isobutyl-Acetate	0.07	±	0.02	0.11	±	0.03	0.09	±	0.02
Isobutyric_acid	1.33	±	0.13	1.91	±	0.05	1.26	±	0.50
Iso-Valeric_Acid	1.07	±	0.06	1.18	±	0.05	1.17	±	0.37
n-propanol	229.17	±	10.36	82.26	±	1.31	196.07	±	25.85
Octanoic_Acid	1.19	±	0.03	1.36	±	0.08	1.31	±	0.48
Pentanol	0.31	±	0.01	0.29	±	0.02	0.33	±	0.02
Propionic_Acid	6.16	±	0.54	1.96	±	0.09	3.62	±	1.12
Valeric_Acid	0.73	±	0.04	0.51	±	0.03	0.58	±	0.15

As was observed in Shiraz wines, GC-FID analyses showed that both commercial references WE372 (33.56 ± 3.45 mg/L) and MERIT (33.88 ± 0.24 mg/L) produced 2016 Merlot wines with noticeably more ethyl acetate, the main ester compound associated with 'apple', 'pineapple', and 'fruity' aromas, than that produced by ARC Nvbij 6 (25.66 ± 2.20 mg/L) (Table 7). Ethyl acetate concentrations in all 2016 Merlot wines also exceeded its sensory detection threshold of 12 mg/L (Rossouw, 2009; Lapalus 2016). However, only WE372 produced wines with a positive association with the 'fruit' aroma based on the descriptive sensory evaluation (Fig. 4e). ARC Nvbij 6 (278 ± 32.62 mg/L) produced 2016 Merlot wines with noticeably less acetic acid, the main volatile acid (imparts unpleasant vinegar off-odours), than that produced by both WE372 (408.40 ± 23.47 mg/L) and MERIT (452.14 ± 38.02 mg/L), respectively (Table 7). This observation, therefore, complements previous reports that higher ethyl acetate levels accentuate VA, as it is the second highest contributor after acetic acid. This observation complements FTIR analyses, as the ARC Nvbij 6 produced wines had a negative association with volatile acidity (Fig. 4b). The same observation was made in all Shiraz wines.

The yeast MERIT (0.71 ± 0.08 mg/L) produced 2016 Merlot wines with noticeably more 2-phenylacetate, which also imparts 'floral' aromas (Lapalus, 2016) than wines produced by WE372 (0.62 ± 0.07 mg/L) and ARC Nvbij 6 (0.60 ± 0.04 mg/L), respectively (Table 7). This observation was complemented by descriptive sensory analyses as MERIT produced wines with a positive association with 'floral' and 'violet' aromas (Fig. 4e). All yeast strains also produced 2016 Merlot wines with comparable ethyl caprate levels, another compound associated with 'floral' below its sensory detection threshold (0.2 mg/L). It can therefore be concluded that this compound did not contribute to 'floral' aromas perceived in this study.

Both MERIT (1.39 ± 0.13 mg/L) and WE372 (1.23 ± 0.13 mg/L) produced 2016 Merlot wines with diethyl succinate levels that exceeded its aroma threshold (1.2 mg/L) (Rossouw, 2009; Lapalus 2016). However, this compound was reported not to contribute individually to wine 'berry-like' or 'fruity' aromas, but synergistically enhances wine aroma due to effect of other aroma compounds (Cortés-Diéguez et al., 2015). Although diethyl succinate in ARC Nvbij 6 (0.70 mg/L ± 0.06 mg/L) produced wines also exceeded the aroma threshold, 2016 Merlot wines were not

perceived to be ‘berry-like’ or ‘fruity’ (Fig. 5e). However, unlike the ARC Nvbij 6 produced Shiraz wines (Fig. 4d & 5d), the ARC Nvbij 6 produced Merlot wines had a negative association with ‘spicy’ aromas that can be associated with a typical varietal Merlot wine. All yeast strains also produced 2016 Merlot wines with comparable ethyl-3-hydroxybutanoate (imparts ‘red berry’ aromas) levels. However, it was below its olfaction perception threshold (Pineau et al., 2009), and it can be accepted that this compound did not contribute to wine aroma during this study. Nonetheless, WE372 produced 2016 Merlot wines had a positive association with ‘red fruit’ or ‘berry’ aroma (Fig. 4e), which can be ascribed to other aroma compounds known to enhance this nuances that were above their olfaction perception threshold.

In terms of higher alcohols, both commercial references MERIT (89.92 ± 12.53 mg/L) and WE372 (81.20 ± 5.89 mg/L) produced 2016 Merlot wines with noticeably more 2-phenyl ethanol (imparts honey-like and rose aromas) (Musarurwa et al., 2016), than that produced with ARC Nvbij 6 (65.03 ± 5.51 mg/L) (Table 7). Furthermore, descriptive sensory evaluation complements this observation, as the 2016 Merlot wines produced with MERIT had the best association with ‘floral’ (rose) aroma (Fig. 4e). Additionally, ARC Nvbij 6 (291.33 ± 23.20 mg/L) produced 2016 Merlot wines with noticeably less isoamyl alcohol (can impart unpleasant solvent off-odours) than that produced by WE372 (332.73 ± 30.56 mg/L) and MERIT (422.23 ± 55.97 mg/L), respectively (Table 7). However, all wines had sought-after aroma nuances (Fig. 4e). Overall, the yeast ARC Nvbij 6 produced noticeably less of the unwanted compounds. This is a positive observation, as excessive levels of aforementioned metabolites will have a negative effect on wine sensory quality.

Table 7. Major wine volatile aroma compounds, namely esters, higher alcohols and fatty acids measured using gas chromatography of small-scale 2016 Merlot wines produced at the Nietvoorbij research cellar following fermentation with a natural experimental yeast strain ARC Nvbij 6 and commercial yeast strains MERIT and WE372. Average values of triplicate fermentations.

Aroma compound (mg/L)	WE372			MERIT			ARC Nvbij 6		
2-Phenyl_Ethanol	81.20	±	5.89	89.92	±	12.53	65.03	±	5.51
2-Phenylethyl_Acetate	0.62	±	0.07	0.71	±	0.08	0.60	±	0.04
3-ethoxy-1-propanol	3.94	±	0.21	0.57	±	0.04	2.32	±	0.18
3-methyl-1-pentanol	3.47	±	0.11	1.38	±	0.15	1.18	±	0.05
4-methyl-1-pentanol	0.37	±	0.04	0.27	±	0.03	0.29	±	0.02
Acetic_Acid	408.40	±	23.47	452.14	±	38.03	278.20	±	32.62
Acetoin	0.37	±	0.01	0.28	±	0.02	0.32	±	0.02
Butanol	2.63	±	0.16	1.17	±	0.11	2.21	±	0.07
Butyric_Acid	0.07	±	0.03	0.15	±	0.07	0.11	±	0.06
Diethyl_Succinate	1.23	±	0.13	1.39	±	0.13	0.70	±	0.06
Ethyl_Acetate	33.56	±	3.45	33.88	±	0.24	25.66	±	2.20
Ethyl_butyrate	0.18	±	0.03	0.17	±	0.02	0.16	±	0.04
Ethyl_Caprate	0.09	±	0.01	0.07	±	0.00	0.08	±	0.01
Ethyl_Caprylate	0.17	±	0.01	0.18	±	0.02	0.20	±	0.03
Ethyl_Hexanoate	0.00	±	0.00	0.01	±	0.01	0.01	±	0.01
Ethyl_Lactate	17.28	±	0.53	6.85	±	0.74	5.89	±	0.24
ethyl_phenylacetate	1.71	±	0.07	1.73	±	0.07	1.69	±	0.05
Ethyl-3-hydroxybutanoate	0.44	±	0.03	0.43	±	0.04	0.38	±	0.08
Hexanoic_Acid	10.92	±	3.59	8.43	±	4.29	8.30	±	4.04
Hexanol	2.88	±	0.33	3.32	±	0.31	3.21	±	0.28
Hexyl_Acetate	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Isoamyl_Acetate	2.21	±	0.19	2.52	±	0.29	1.56	±	0.18
Isoamyl_alcohol	332.73	±	30.56	422.23	±	55.97	291.33	±	23.20
Isobutanol	44.55	±	4.09	96.89	±	11.73	38.25	±	2.44
Isobutyl-Acetate	0.04	±	0.03	0.12	±	0.01	0.00	±	0.00
Isobutyric_acid	2.37	±	0.25	3.90	±	0.44	1.58	±	0.07
Iso-Valeric_Acid	2.86	±	0.27	3.52	±	0.49	2.56	±	0.24
n-propanol	96.54	±	6.59	38.56	±	0.49	68.83	±	3.65
Octanoic_Acid	1.33	±	0.12	1.44	±	0.16	1.50	±	0.07
Pentanol	0.00	±	0.00	0.00	±	0.00	0.29	±	0.01
Propionic_Acid	2.66	±	0.21	0.87	±	0.03	1.66	±	0.13
Valeric_Acid	1.28	±	0.02	0.94	±	0.07	1.36	±	0.12

As was observed in the 2016 Merlot and Shiraz as well as 2017 Shiraz wines, GC-FID analyses showed that MERIT (95.63 ± 9.78 mg/L) produced 2017 Merlot wines with noticeably more ethyl acetate, the main ester (imparts 'fruity' aromas), than wines produced by ARC Nvbij 6 (83.27 ± 4.00 mg/L) (Table 8). The yeast MERIT is, therefore a good producer of said aroma compound. However, ARC Nvbij 6 produced wines with a higher concentration of ethyl acetate than wines produced with WE372 (66.92 ± 18.35 mg/L). Ethyl acetate concentrations in all 2017 Merlot wines also exceeded its sensory detection threshold of 12 mg/L (Rossouw, 2009; Lapalus 2016). However, only WE372 produced wines with a positive association with 'red fruit' or 'berry' aroma based on the descriptive sensory evaluation (Fig. 5e). Furthermore, WE372 (10.17 ± 2.41 mg/L) produced 2017 Merlot wines with noticeably more ethyl lactate, associated with malo-lactic fermentation (Cortés-Diéguez et al., 2015), than wines produced by ARC Nvbij 6 (4.39 ± 0.33 mg/L) and MERIT (3.21 ± 0.42 mg/L), respectively (Table 8). This observation complements previous studies and the yeast manufacturer's recommendation that WE372 has a stimulatory effect on malo-lactic fermentation (Schöltz, 2013). The yeast ARC Nvbij 6 (0.39 ± 0.02 mg/L) produced 2017 Merlot wines with noticeably more 2-phenylethyl acetate (imparts 'floral' aromas) (Lapalus, 2016), than wines produced by WE372 (0.34 ± 0.07 mg/L) and MERIT (0.29 ± 0.05 mg/L), respectively (Table 8). However, WE372 produced wines with the strongest association with 'floral' aroma (Fig. 5e). All yeast strains also produced 2017 Merlot wines with ethyl-3-hydroxybutanoate (imparts 'red berry' aromas) levels below its olfaction perception threshold (Pineau et al., 2009), and it can be accepted that this compound did not contribute to wine aroma during this study. Nonetheless, WE372 produced 2017 Merlot wines had a positive association with 'red fruit' or 'berry' aroma (Fig. 5e). A similar observation was made for the 2016 Merlot wines (Fig. 4e).

In terms of fatty acids, as was observed in 2016 Merlot and Shiraz as well as 2017 Shiraz wines the ARC Nvbij 6 (521.72 ± 31.65 mg/L) produced 2017 Merlot wines with noticeably less acetic acid, the main volatile acid (imparts unpleasant vinegar off-odours) than that produced by both MERIT (779.79 ± 67.39 mg/L) and WE372 (798,82± 294.48mg/L), respectively (Table 8). This observation complements FTIR analyses, as the ARC Nvbij 6 produced wines had a negative

association with volatile acidity; whilst WE372 produced wines had a positive association with volatile acidity (Fig. 5e). This study therefore showed that the experimental yeast strain is a low volatile acidity producer in two cultivars *i.e.* Shiraz and Merlot and across both vintages *i.e.* 2016 and 2017. All yeast strains also produced 2017 Merlot wines with comparable ethyl caprate levels (imparts 'floral') below its sensory detection threshold (0.2 mg/L), and its effect on 2017 Merlot wine sensory quality is negligible. Only MERIT (2.14 ± 1.55 mg/L) produced 2017 Merlot wines with diethyl succinate (1.2 mg/L) levels that exceeded its aroma threshold (Rossouw, 2009; Lapalus 2016), but as with Shiraz wines it does not to contribute individually to wine 'berry-like' or 'fruity' aroma (Cortés-Diéguez et al., 2015). However, WE372 was the only strain to have produced 2017 Merlot wines with a positive association with 'floral' aroma (Fig. 5e), despite producing this compound below its sensory detection threshold. It can be envisaged that other aroma compounds associated with this wine attribute, were the final effectors in this regard. The yeast MERIT produced 2017 Merlot wines had a positive association with 'spicy' aromas that can be associated with a typical varietal Merlot wine. However, none of the aroma compounds were previously reported to associate with this Merlot wine attribute.

In terms of higher alcohols, ARC Nvbij 6 (52.01 ± 4.07 mg/L) produced 2017 Merlot wines with noticeably less 2-phenyl ethanol (imparts honey-like and rose aromas) (Musarurwa et al., 2016), than that produced with WE372 (58.77 ± 9.56 mg/L) and more than MERIT produced wines (41.28 ± 5.46 mg/L), respectively (Table 8). However, descriptive sensory evaluation showed that the 2017 Merlot wines produced with WE372 had the best association with 'floral' (rose) aroma (Fig. 5e). Additionally, ARC Nvbij 6 (299.20 ± 12.81 mg/L) produced 2017 Merlot wines with noticeably less isoamyl alcohol (can impart unpleasant solvent off-odours) than that produced by WE372 (305.19 ± 66.99 mg/L) (Table 8). Indications, therefore are that elevated isoamyl alcohol masked the effect of the aroma-enhancing metabolites, hence ARC Nvbij 6 produced wines had a negative association with sought-after aromas (Fig. 5e).

Table 8. Major wine volatile aroma compounds, namely esters, higher alcohols and fatty acids measured using gas chromatography of small-scale 2017 Merlot wines produced at the Nietvoorbij research cellar following fermentation with a natural experimental yeast strain ARC Nvbij 6 and commercial yeast strains MERIT and WE372. Average values of triplicate fermentations.

Aroma compounds (mg/L)	WE372			MERIT			ARC Nvbij 6		
2-Phenyl_Ethanol	58.45	±	20.11	41.28	±	5.46	52.01	±	4.07
2-Phenylethyl_Acetate	0.34	±	0.07	0.29	±	0.05	0.39	±	0.02
3-ethoxy-1-propanol	8.00	±	2.84	4.45	±	1.16	8.55	±	0.26
3-methyl-1-pentanol	2.05	±	0.48	0.69	±	0.06	0.88	±	0.07
4-methyl-1-pentanol	0.18	±	0.12	0.00	±	0.00	0.22	±	0.00
Acetic_Acid	798,82	±	294.48	779.79	±	67.39	521.72	±	31.65
Acetoin	0.29	±	0.07	0.22	±	0.01	0.25	±	0.00
Butanol	1.90	±	0.56	1.12	±	0.10	1.62	±	0.02
Butyric_Acid	0.01	±	0.02	0.00	±	0.00	0.00	±	0.00
Diethyl_Succinate	2.14	±	1.55	0.78	±	0.10	0.85	±	0.08
Ethyl_Acetate	66.92	±	18.35	95.63	±	9.78	83.27	±	4.00
Ethyl_butyrate	0.17	±	0.05	0.10	±	0.01	0.12	±	0.01
Ethyl_Caprate	0.07	±	0.03	0.02	±	0.01	0.04	±	0.00
Ethyl_Caprylate	0.09	±	0.10	0.01	±	0.01	0.05	±	0.02
Ethyl_Hexanoate	0.03	±	0.05	0.00	±	0.00	0.00	±	0.00
Ethyl_Lactate	10.17	±	2.41	3.21	±	0.42	4.39	±	0.33
ethyl_phenylacetate	0.92	±	0.17	0.73	±	0.01	0.73	±	0.06
Ethyl-3-hydroxybutanoate	0.40	±	0.05	1.23	±	0.75	0.17	±	0.00
Hexanoic_Acid	2.93	±	4.15	6.52	±	4.67	2.63	±	3.17
Hexanol	3.03	±	1.73	1.81	±	0.19	1.93	±	0.12
Hexyl_Acetate	0.00	±	0.00	0.00	±	0.00	0.11	±	0.01
Isoamyl_Acetate	0.88	±	0.35	0.53	±	0.03	0.62	±	0.05
Isoamyl_alcohol	305.19	±	66.99	244.88	±	24.90	299.20	±	12.81
Isobutanol	53.57	±	6.03	81.65	±	2.58	69.26	±	5.04
Isobutyl-Acetate	0.02	±	0.03	0.10	±	0.00	0.09	±	0.01
Isobutyric_acid	3.05	±	0.62	3.79	±	0.05	3.27	±	0.41
Iso-Valeric_Acid	2.89	±	1.06	1.47	±	0.29	2.20	±	0.14
n-propanol	139.64	±	25.97	103.65	±	21.13	178.43	±	9.79
Octanoic_Acid	0.98	±	0.62	0.46	±	0.14	0.73	±	0.06
Pentanol	0.31	±	0.02	0.31	±	0.02	0.31	±	0.01
Propionic_Acid	2.59	±	0.19	1.45	±	0.28	2.44	±	0.09
Valeric_Acid	1.42	±	0.46	0.08	±	0.00	1.04	±	0.03

As was observed in Merlot and Shiraz during the 2016 and 2017 vintages, GC-FID analyses showed that the MERIT (42.65 ± 4.98 mg/L) produced 2016 Cabernet Sauvignon wines with noticeably more ethyl acetate, the main ester (also imparts 'apple' aromas) than wines produced by ARC Nvbij 6 (37.13 ± 7.84 mg/L) and WE372 (33.45 ± 5.24 mg/L), respectively (Table 9). Descriptive sensory evaluation complements this observation, as only MERIT produced 2016 Cabernet Sauvignon wines with a positive association with 'vegetative fresh' (green apple) aroma (Fig. 4f). Nonetheless, all yeast consistently produced wines with ethyl acetate levels above its sensory detection threshold of 12 mg/L (Rossouw, 2009; Lapalus 2016). Indications, therefore are that other compounds masked its effect in those wines lacking 'vegetative green' aromas. The WE372 (29.60 ± 1.84 mg/L) produced 2016 Cabernet Sauvignon wines with noticeably more ethyl lactate, associated with malo-lactic fermentation (Cortés-Diéguez et al., 2015) than wines produced by MERIT (18.04 ± 1.14 mg/L) and ARC Nvbij 6 (14.22 ± 3.31 mg/L), respectively (Table 9). This observation complements observation made in Merlot and Shiraz during the 2016 and 2017 vintages as well as the yeast manufacturer's recommendation that WE372 has a stimulatory effect on malo-lactic fermentation. The yeast ARC Nvbij 6 (0.52 ± 0.08 mg/L) produced 2016 Cabernet Sauvignon wines with marginally more 2-phenylacetate (imparts 'floral' aromas) (Lapalus, 2016), than wines produced with MERIT (0.51 ± 0.05 mg/L) and WE372 (0.34 ± 0.03 mg/L), respectively (Table 9). However, 'floral' aromas are not usually associated with Cabernet Sauvignon wines; hence, this parameter was excluded from descriptive sensory evaluation aroma descriptors (Fig. 4f). All yeast strains also produced 2016 Cabernet Sauvignon wines with ethyl-3-hydroxybutanoate (imparts 'red berry' aromas) levels below its olfaction perception threshold (Pineau et al., 2009), as was observed in Shiraz and Merlot wines. Nonetheless, MERIT produced 2016 Cabernet Sauvignon wines had a stronger association with 'red fruit' or 'berry' aroma (Fig. 4f). Aroma compounds present above their olfaction perception threshold that is also known to enhance this aroma was, therefore, final effectors.

All yeast strains produced 2016 Cabernet Sauvignon wines with diethyl succinate levels (Table 9) that exceeded its aroma threshold, however none of the wines had a positive association 'berry-like' aroma (Cortés-Diéguez et al., 2015). As, Cabernet Sauvignon is renowned for other

compounds *i.e.* methoxypyrazines, which imparts vegetative aromas (Hart et al., 2016), they could have masked the effect of the ester compounds. Unfortunately, this study had no access to state of art equipment that is able to measure these methoxypyrazines *i.e.* 2-methoxy-3-isobutylmethoxypyrazine. Nonetheless, all yeast strains produced typical Cabernet Sauvignon wines as vegetative sensory attributes and/or nuances were perceived by sensory evaluation panel (Fig. 4f).

In terms of fatty acids, as was observed in Merlot and Shiraz during the 2016 and 2017 vintages the ARC Nvbij 6 (335.22 ± 10.89 mg/L) produced 2016 Cabernet Sauvignon wines with noticeably less acetic acid, the main volatile acid than WE372 (408.86 ± 14.21 mg/L) and MERIT (505.67 ± 33.15 mg/L), respectively (Table 9). This observation complements FTIR analyses, as the WE372 produced wines had the strongest association with volatile acidity, whilst ARC Nvbij 6 and MERIT produced wines had a negative association with volatile acidity (Fig. 4c). This study, therefore, showed this the experimental yeast strain is low volatile acidity producer in three popular red cultivars *i.e.* Shiraz, Merlot and Cabernet Sauvignon.

Overall, WE372 (50.34 ± 1.67 mg/L) produced 2016 Cabernet Sauvignon wines with noticeably less 2-phenyl ethanol (imparts honey-like and rose aromas) (Musarurwa et al., 2016), than wines produced with MERIT (62.86 ± 2.90) and ARC Nvbij 6 (62.43 ± 10.43 mg/L), respectively (Table 9). However, the 'floral' aroma was not included in the list of Cabernet Sauvignon aroma descriptors, as it is not associated with this cultivar. Furthermore, even if it was included the sensory detection thresholds of methoxypyrazines is much lower than most ester compounds (Marais, 1994; Lapalus, 2016). This aspect will be addressed as part of another parallel study. Both, WE372 (263.24 ± 7.86 mg/L) and ARC Nvbij 6 (312.73 ± 31.06 mg/L) produced 2016 Cabernet Sauvignon wines with noticeably **less** isoamyl alcohol (can also masked varietal aroma) than MERIT (350.50 ± 6.50 mg/L) (Table 9). However, the MERIT produced wines had a more positive association with varietal sensory attributes (Fig. 4f).

Table 9. Major wine volatile aroma compounds, namely esters, higher alcohols and fatty acids measured using gas chromatography of small-scale 2016 Cabernet Sauvignon wines produced at the Nietvoorbij research cellar following fermentation with a natural experimental yeast strain ARC Nvbij 6 and commercial yeast strains MERIT and WE372. Average values of triplicate fermentations.

Aroma compound (mg/L)	WE372			MERIT			ARC Nvbij 6		
2-Phenyl_Ethanol	50.34	±	1.67	62.86	±	2.90	62.43	±	10.41
2-Phenylethyl_Acetate	0.34	±	0.03	0.51	±	0.05	0.52	±	0.08
3-ethoxy-1-propanol	2.83	±	0.09	0.66	±	0.03	1.89	±	0.56
3-methyl-1-pentanol	5.95	±	0.37	3.63	±	0.23	2.86	±	0.66
4-methyl-1-pentanol	0.28	±	0.01	0.22	±	0.00	0.30	±	0.03
Acetic_Acid	408.86	±	14.21	505.67	±	33.15	335.22	±	10.89
Acetoin	0.30	±	0.01	0.25	±	0.00	0.32	±	0.04
Butanol	2.15	±	0.14	1.22	±	0.05	1.99	±	0.46
Butyric_Acid	0.02	±	0.02	0.26	±	0.11	0.27	±	0.16
Diethyl_Succinate	7.40	±	0.19	13.75	±	1.81	7.36	±	2.91
Ethyl_Acetate	33.45	±	5.24	42.65	±	4.98	37.13	±	7.84
Ethyl_butyrate	0.15	±	0.03	0.16	±	0.04	0.19	±	0.03
Ethyl_Caprate	0.06	±	0.01	0.05	±	0.00	0.07	±	0.02
Ethyl_Caprylate	0.08	±	0.01	0.18	±	0.02	0.20	±	0.03
Ethyl_Hexanoate	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Ethyl_Lactate	29.60	±	1.84	18.04	±	1.14	14.22	±	3.31
Ethyl_phenylacetate	1.83	±	0.05	2.01	±	0.01	1.90	±	0.19
Ethyl-3-hydroxybutanoate	0.56	±	0.03	0.48	±	0.02	0.42	±	0.08
Hexanoic_Acid	8.18	±	0.79	1.03	±	1.46	2.12	±	3.00
Hexanol	1.46	±	0.04	1.77	±	0.15	1.81	±	0.22
Hexyl_Acetate	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Isoamyl_Acetate	0.82	±	0.12	1.64	±	0.27	1.31	±	0.31
Isoamyl_alcohol	263.24	±	7.68	350.50	±	6.50	312.73	±	31.06
Isobutanol	40.46	±	1.70	90.56	±	6.53	45.95	±	7.48
Isobutyl-Acetate	0.02	±	0.03	0.09	±	0.01	0.02	±	0.03
Isobutyric_acid	2.03	±	0.08	3.49	±	0.39	1.71	±	0.34
Iso-Valeric_Acid	2.02	±	0.07	2.45	±	0.20	2.19	±	0.31
n-propanol	62.41	±	0.61	30.63	±	0.87	48.63	±	10.02
Octanoic_Acid	0.90	±	0.10	1.28	±	0.15	1.47	±	0.18
Pentanol	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Propionic_Acid	0.86	±	0.00	0.34	±	0.04	1.00	±	0.29
Valeric_Acid	1.26	±	0.04	1.02	±	0.03	1.31	±	0.18

As was observed in Shiraz, Merlot and Cabernet Sauvignon wines during the 2016 and 2017 vintages, GC-FID analyses showed that the MERIT (75.82 ± 7.83 mg/L) produced 2017 Cabernet Sauvignon wines with noticeably more ethyl acetate, the main ester (also imparts 'apple' aromas), than wines produced by ARC Nvbij 6 (52.37 ± 8.36 mg/L) and WE372 (45.77 ± 2.02 mg/L), respectively (Table 10). Descriptive sensory evaluation, again complements this observation, as only MERIT produced 2017 Cabernet Sauvignon wines with a positive association with 'vegetative fresh' (green apple) aroma (Fig. 5f). Nonetheless, all yeast consistently produced wines with ethyl acetate levels above its sensory detection threshold of 12 mg/L (Rossouw, 2009; Lapalus 2016). Indications therefore are that methoxypyrazines masked the effect of this ester compound. Both WE372 (16.81 ± 0.12 mg/L) and MERIT (16.35 ± 1.11 mg/L) produced 2017 Cabernet Sauvignon wines with noticeably more ethyl lactate, associated with malo-lactic fermentation (Cortés-Diéguez et al., 2015) than wines produced by and ARC Nvbij 6 (12.31 ± 2.22 mg/L) (Table 10). This observation complements observation made in Shiraz, Merlot and Cabernet Sauvignon wines during the 2016 and 2017 vintages, that WE372 has a stimulatory effect on malolactic fermentation. However, the fact that this compound was also detected in the MERIT and ARC Nvbij 6 produced wines, suggests that they are also stimulators of malolactic fermentation, albeit at to a lesser extent than WE372.

The yeast MERIT (75.82 ± 7.83 mg/L) produced 2017 Cabernet Sauvignon wines with noticeably more ethyl acetate, the main ester (imparts 'red fruit' aromas) than wines produced by WE372 (45.77 ± 2.02 mg/L) and ARC Nvbij 6 (0.15 ± 0.01 mg/L), respectively (Table 10). Even though the levels were below its sensory detection threshold, the WE372 produced Cabernet Sauvignon wines were perceived to have red fruit (berry) aroma (Fig. 5f). All yeast strains produced 2017 Cabernet Sauvignon wines with diethyl succinate levels (Table 10) that exceeded its aroma threshold, however only WE372 had a positive association 'berry-like' aroma (Cortés-Diéguez et al., 2015). Indications therefore are that diethyl succinate is a stronger effector than ethyl acetate with regard to 'red fruit' aromas.

In terms of fatty acids, as was observed in Merlot and Shiraz during the 2016 and 2017 vintages ARC Nvbij 6 (117.67 ± 8.47 mg/L) produced 2017 Cabernet Sauvignon wines with

noticeably less acetic acid, the main volatile acid than MERIT (275.40 ± 19.29 mg/L) and WE372 (358.20 ± 30.52 mg/L), respectively (Table 10). This observation complements FTIR analyses, as the ARC Nvbij 6 produced wines had a negative association with volatile acidity and total acidity, whilst WE372 and MERIT produced wines had a positive association with volatile acidity and total acidity, respectively (Fig. 5f). This study, therefore, repeatedly showed the experimental yeast strain is a low volatile acidity producer in three popular red cultivars *i.e.* Shiraz, Merlot, and Cabernet Sauvignon (Fig. 4a, 4b, 4c, 5a, 5b, & 5c).

Overall, ARC Nvbij 6 (45.32 ± 8.55 mg/L) produced 2017 Cabernet Sauvignon wines with noticeably more 2-phenyl ethanol (imparts honey-like and rose aromas) (Musarurwa et al., 2016), than wines produced with MERIT (38.28 ± 3.88) and WE372 (31.96 ± 2.51 mg/L), respectively (Table 10). As 'floral' and/or 'honey' aromas were not included in the list of Cabernet Sauvignon aroma descriptors, as it is not associated with this cultivar, it can be speculated that the panel perceived this metabolite as 'vegetative cooked' in the ARC Nvbij 6 produced wines (Fig. 5f) However, methoxypyrazines are known to impart vegetative cooked (asparagus) and vegetative fresh (grass, green apple, green pepper) aromas (Marais, 1994; Lapalus, 2016). This aspect will be addressed as part of another parallel study. Both, WE372 (211.02 ± 11.32 mg/L) and MERIT (269.92 ± 38.73 mg/L) produced 2017 Cabernet Sauvignon wines with noticeably less isoamyl alcohol (can also masked varietal aroma) than ARC Nvbij 6 (346.03 ± 21.82 mg/L) (Table 10). This observation might also declare why wines produced by the latter, were perceived to be 'vegetative cooked' by descriptive sensory panel (Fig. 5f). Nonetheless, ARC Nvbij 6 proved that it has a role to play in the production of varietal red wines based on the chemical, sensory, and metabolic attributes of Shiraz, Merlot and Cabernet Sauvignon wines. Futhermore, this study showed that the experimental yeast produced less aroma compounds associated with fruity Shiraz, Merlot, and Cabernet Sauvignon aroma notes compared to WE372 and MERIT (Appendix II, Fig.4 & 5). Nonetheless, ARC Nvbij 6 consistently produce less of the undesirable compounds that are associated with wine off-odours, which can influence wine sensory quality negatively. These off-odours are also known to mask the effect of the sought-after compounds associated with 'fruity' aroma and flavour.

Table 10. Major wine volatile aroma compounds, namely esters, higher alcohols and fatty acids measured using gas chromatography of small-scale 2017 Cabernet Sauvignon wines produced at the Nietvoorbij research cellar following fermentation with a natural experimental yeast strain ARC Nvbij 6 and commercial yeast strains MERIT and WE372. Average values of triplicate fermentations.

Aroma compounds (mg/L)	WE372			MERIT			ARC Nvbij 6		
2-Phenyl_Ethanol	31.96	±	2.51	38.28	±	3.88	45.32	±	8.55
2-Phenylethyl_Acetate	0.30	±	0.04	0.33	±	0.06	0.38	±	0.07
3-ethoxy-1-propanol	8.27	±	0.28	4.65	±	0.69	6.28	±	0.71
3-methyl-1-pentanol	3.38	±	0.02	3.29	±	0.22	2.47	±	0.45
4-methyl-1-pentanol	0.16	±	0.11	0.00	±	0.00	0.19	±	0.13
Acetic_Acid	358.20	±	30.52	275.40	±	19.29	117.67	±	8.47
Acetoin	0.24	±	0.01	0.21	±	0.01	0.25	±	0.02
Butanol	1.02	±	0.08	0.97	±	0.13	1.28	±	0.27
Butyric_Acid	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Diethyl_Succinate	1.42	±	0.14	2.21	±	0.35	1.68	±	0.34
Ethyl_Acetate	45.77	±	2.02	75.82	±	7.83	52.37	±	8.36
Ethyl_butyrate	0.12	±	0.03	0.13	±	0.04	0.12	±	0.04
Ethyl_Caprate	0.04	±	0.01	0.03	±	0.02	0.05	±	0.02
Ethyl_Caprylate	0.06	±	0.01	0.08	±	0.03	0.10	±	0.03
Ethyl_Hexanoate	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Ethyl_Lactate	16.81	±	0.12	16.35	±	1.11	12.31	±	2.22
Ethyl_phenylacetate	0.39	±	0.01	0.40	±	0.02	0.40	±	0.03
Ethyl-3-hydroxybutanoate	0.36	±	0.07	0.17	±	0.02	0.15	±	0.01
Hexanoic_Acid	14.79	±	4.85	0.79	±	1.12	7.27	±	10.29
Hexanol	1.20	±	0.12	1.62	±	0.25	1.59	±	0.32
Hexyl_Acetate	0.10	±	0.01	0.13	±	0.01	0.09	±	0.06
Isoamyl_Acetate	0.62	±	0.09	0.65	±	0.12	0.68	±	0.12
Isoamyl_alcohol	211.02	±	11.32	269.92	±	38.73	346.03	±	21.82
Isobutanol	47.01	±	1.72	93.75	±	6.24	67.67	±	12.89
Isobutyl-Acetate	0.00	±	0.00	0.06	±	0.05	0.07	±	0.01
Isobutyric_acid	2.46	±	0.15	3.60	±	0.35	2.62	±	0.57
Iso-Valeric_Acid	1.83	±	0.25	1.43	±	0.25	2.07	±	0.42
n-propanol	184.45	±	13.62	149.94	±	9.38	210.79	±	33.17
Octanoic_Acid	0.85	±	0.11	0.94	±	0.21	1.07	±	0.17
Pentanol	0.00	±	0.00	0.08	±	0.12	0.19	±	0.14
Propionic_Acid	1.91	±	0.28	1.04	±	0.16	1.52	±	0.33
Valeric_Acid	0.84	±	0.04	0.60	±	0.03	0.88	±	0.13

3.4.6 Gas chromatography- mass spectrometry (GC-MS) analysis of volatile thiols

It is well known within the wine biotechnology community that odourless grape-derived precursors such as cysteinylated conjugates are converted into aroma active compounds known as volatile thiols, namely 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA) (Keyzers and Boss 2010, Dennis et al. 2012). These volatile thiols were thus measured for in Shiraz, Merlot and Cabernet Sauvignon wines produced using the commercial reference yeasts *i.e.* MERIT and WE372, and the experimental yeast *i.e.* ARC Nvbij 6 using a GC-MS upon completion of fermentation. It should be noted that Renault et al. (2016) used a new convention by referring to 4MMP, 3MH, and 3MHA as 4-methyl-4-sulfanylpentan-2-one (4MSP), 3-sulfanylhexan-1-ol (3SH), and 3-sulfanylhexyl acetate (3SHA), respectively. Nonetheless, the former conventions were used in this study, as *e.g.* 3-mercaptohexan-1-ol (3MH) and 3-sulfanylhexan-1-ol (3SH) are still the same chemical compounds (Luisier et al., 2008).

Although extensive research has been conducted on the volatile thiols in the white cultivars especially Sauvignon blanc, limited research has focused on the red cultivars. Subsequently, GC-MS analyses were conducted to add value to this aspect. The GC-MS analyses showed that 3MHA, which imparts passion-fruit, grapefruit and guava aromas (Tominaga et al., 1998a,b; Dubourdieu et al., 2006 Tominaga et al., 2006), could not be detected in any of the 2016 Shiraz wines, (Fig. 6a). However, the experimental yeast *i.e.* ARC Nvbij 6 produced 2016 Shiraz wines with more 3MH (imparts blackcurrant aromas in red wines) (Blanchard et al., 2000), than both the commercial reference yeasts *i.e.* MERIT and WE372, respectively. This is in agreement with Swiegers et al. (2005) as the authors also found that different yeast strains had differentiating abilities to convert 3MH to 3MHA. It was previously reported that some wine yeast strains, referred to as “3MH converters” convert 3MH to 3MHA through their metabolic activity (Swiegers et al., 2006, 2007a). Indications therefore are that none of the yeast strains managed to convert 3MH. These observations will be investigated further as part of another study. It was also observed that MERIT (396.83 mg/L) released the most 4MMP (imparts box tree, passion fruit, and blackcurrant aromas) followed by ARC Nvbij 6 (292.57 mg/L) and WE372 (85.03 mg/L) in the 2016 Shiraz

wines. However, descriptive sensory evaluation showed that the ARC Nvbij 6 produced wines had a stronger association with the berry aroma (Fig. 4d), which suggests other thiols *e.g.* 3MH could have as well as ester compounds previously mentioned also enhanced 'red fruit' (berry) aroma.

As was observed during the 2016 harvest, the yeast WE372 again failed to convert 3MH, as the 2017 Shiraz wines had no 3MHA (Fig. 7a). However, MERIT (54.05 mg/L) and ARC Nvbij 6 (6.29 mg/L) produced 2017 Shiraz wines showed low levels of 3MHA but yet above sensory detection threshold as described by Swiegers et al. (2007b). However, descriptive sensory evaluation showed that MERIT produced Shiraz wines had a negative association with 'red fruit' (berry) aroma (Fig. 4d). In terms of 3MH, MERIT (566.9 mg/L) released higher concentrations in the 2017 Shiraz compared to ARC Nvbij 6 (510.84 mg/L) and WE372 (231.66 mg/L), respectively. However, MERIT released the lowest 3MH in the 2016 vintage (Fig. 6a). Nonetheless, vintages are known to affect final wine chemical and sensory attributes. It was again observed that MERIT (2720.92 mg/L) released the most 4MMP (imparts box tree, passion fruit, and black currant aromas) followed by ARC Nvbij 6 (2067.44 mg/L) and WE372 (1220.23 mg/L) in the 2017 Shiraz wines. However, descriptive sensory evaluation showed that only the WE372 produced wines had a positive association with 'berry' aroma (Fig. 4d). The ARC Nvbij 6 produced 2017 Shiraz wines also had a stronger association with 'berry' aroma compared to MERIT (Fig. 4d). These observations complement that of the 2016 vintage and support our notion that other thiols must also enhance 'red fruit' aroma.

It was observed in the 2016 Merlot wines that MERIT (78.36 mg/L) released more 4MMP (imparts box tree, passion fruit, and black currant aromas) than WE372 (70.23 mg/L) and ARC Nvbij 6 (69.77 mg/L), respectively (Fig. 6b). A similar trend was observed with regard to 3MH (associated with citrus aroma), as MERIT (408.47 mg/L) and ARC Nvbij 6 (389.43 mg/L) produced 2016 Merlot wines with noticeably higher levels than WE372 (309.98 mg/L) (Fig. 6b). However, descriptive sensory evaluation showed that MERIT produced 2016 Merlot wines with a positive association with 'floral' and 'violet' aromas (Fig. 4e). These observations suggest that 4MMP and 3MH might also enhance 'floral' and 'violet' aromas. As these volatile compounds, have only recently been reported in red wines, this notion cannot be excluded. However, WE372

(322.01 mg/L) produced 2016 Merlot wines with noticeably more 3MHA (imparts passion-fruit, grapefruit, gooseberry and guava aromas, than ARC Nvbij6 (171.90 mg/L) and MERIT (79.83 mg/L), respectively (Fig. 6b). Therefore, WE372 is higher "3MH converter" in 2016 Merlot wines, and it can tentatively be said that lower 3MH levels observed were due to higher conversation rate by this yeast strain. The descriptive sensory evaluation showed that only WE372 produced 2016 Merlot wines had a positive association with 'berry' and 'fruit' aromas (Fig. 6b), which suggests that 3MHA might also contribute to this sensory attributes alongside esters.

With regard to the 2017 Merlot wines, the experimental yeast ARC Nvbij 6 (2900.17 mg/L) released the highest concentration of 4MMP followed by MERIT (2554.59 mg/L) and WE372 (143.93 mg/L), respectively (Fig. 7b). However, descriptive sensory evaluation showed that the ARC Nvbij 6 produced 2017 Merlot wines had a negative association will all the sought-after sensory attributes (Fig. 4e). As the 4MMP levels were profoundly higher in this wine than its sensory detection threshold (0.06 mg/L), it can be speculated that it was perceived negative. Previous research reported that excessively high thiols are associated with undesirable wine off-odours *e.g.* 'rotten egg' (Swiegers et al., 2007a). A conflicting observation was made for the 2017 Merlot wines compared to the 2016 wines with regards to 3MHA, as none of the yeast strains produced wines with analytical detectable 3MHA levels (Fig. 7b). The experimental yeast ARC Nvbij 6 (708.64 mg/L) released the highest levels of 3MH, than both commercial references MERIT (631.56 mg/L) and WE372 (398.24 mg/L), respectively (Fig. 7b).

The experimental yeast ARC Nvbij 6 (100.73 mg/L) released noticeably more 4MMP in the 2016 Cabernet Sauvignon wines than WE372 (48.79 mg/L) and MERIT (28.78 mg/L), respectively (Fig. 6c). Descriptive sensory evaluation complemented this observation, as only MERIT produced 2016 Cabernet Sauvignon wines had a positive association with 'vegetative fresh' aromas (Fig. 4f). Methoxypyrazines a compound naturally present in Cabernet Sauvignon are associated with 'vegetative' aromas and are known to mask the effect of thiols (Marais, 1994). Both commercial references WE372 (581.02 mg/L) and MERIT (420.53 mg/L) was also shown to release noticeably more 3MH than ARC Nvbij 6 (382.96 mg/L) (Fig. 6c). However, ARC Nvbij 6 (258.84 mg/L) was shown to be a better '3MH converter' than MERIT (90.42 mg/L) (Fig. 6c) and

produce 2016 Cabernet wines with a better overall sensory quality (Fig. 3f). Nonetheless, WE372 was shown to be the best overall '3MH converter' (Fig. 6c).

As was observed in 2016, the yeast ARC Nvbij 6 (2631.06 mg/L) released noticeably more 4MMP in the 2017 Cabernet Sauvignon wines than WE372 (714.77 mg/L) (Fig 9c). However, MERIT (2740.30 mg/L) was the highest 4MMP releaser. The descriptive sensory evaluation showed that ARC Nvbij 6 and MERIT produced 2017 Cabernet Sauvignon wines had a positive association with 'vegetative' aromas (Fig. 4f). It can tentatively be said that elevated 4MMP levels could exacerbate the perceived vegetative aromas, opposed to enhancing the expected and sought-after black currant ('black' fruit) aromas. The yeast ARC Nvbij 6 (1281.18 mg/L) was shown to release noticeably more 3MH than both MERIT (1270.33 mg/L) and WE372 (1148.19 mg/L), respectively (Fig. 7c). Furthermore, ARC Nvbij 6 (39.80 mg/L) was the only strain that converted 3MH into 3MHA (Fig. 7c). This observation complements what was observed in Shiraz wines and even the WE372 produced 2016 Merlot wines and 2017 Cabernet Sauvignon wines, which suggests that ARC Nvbij 6 is a true 3MH and 4MMP releaser. Furthermore, ARC Nvbij 6 was also the only strain to have converted 3MH during both vintages in at least two cultivars. Therefore, this strain has a commercial role to play in the production of varietal red wines.

Overall, all yeast strains exceeded the olfactive perception threshold for 4MMP and 3MH release. Both commercial references also failed to convert 3MH to 3MHA during one vintage in two cultivars. However, where 3MHA was detected it exceeded the aroma threshold. Thus, 4MMP and/or 3MH as well as 3MHA, when it was detected, could have contributed to red wine varietal aromas and flavours. There appears to be a shift between the concentrations of 4MMP and, 3MHA and 3MH in the 2017 harvest season, as 4MMP concentrations were fairly low during 2016 harvest with the exception of Shiraz wines. This is clearly a vintage effect that leads to lower 3MH precursors and increased levels of 4MMP precursors, as the yeast inoculum was the only variable from an oenology perspective. A study themed "liberation of thiols" attracted a lot of attention and reported that different yeast strains differ in their ability to release these compounds from their precursors irrespective of vintage (Subileau et al. 2008; Capone et al. 2010; Winter et al. 2011). However, yeast strains only release a very small portion of thiols, as the

liberation of cysteine-3MH to 3MH (and 3MHA) ranges from 0.1 to 12% with a substantial amount of precursors left unaltered (Subileau et al. 2008). A yeast enzyme, namely carbon-sulfur *β*-lyase is responsible for volatile thiol release (Howell et al. 2005; Swiegers et al. 2007b; Ugliano 2009; Holt et al. 2011). Yeast strains with more carbon-sulfur *β*-lyase activity will release more thiols thus yeast selection can be used as a tool to modulate thiol levels in wine (Dubourdieu et al. 2006, Swiegers and Pretorius 2007, Roncoroni et al. 2011). With this background, another objective of the study was to investigate wine yeast protein expression at the beginning and stationary phases of alcoholic fermentation.

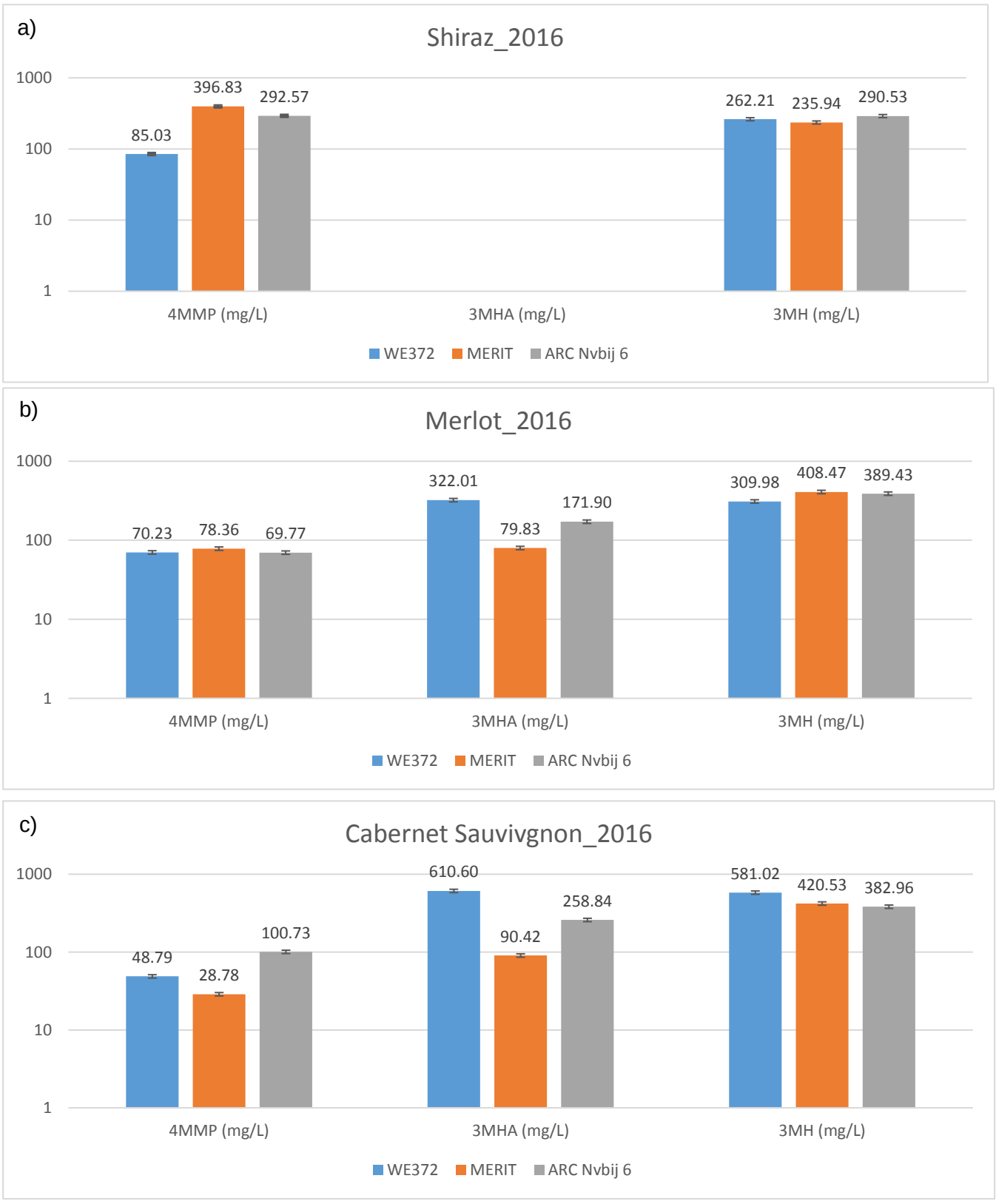

Figure 6. Concentrations of volatile thiols (4-mercapto-4-methylpentan-2-one, 4MMP; 3-mercaptohexan-1-ol, 3MH; and 3-mercaptohexyl acetate, 3MHA) measured using GC-FID for 2016 wines a) Shiraz, b) Merlot, and c) Cabernet Sauvignon.

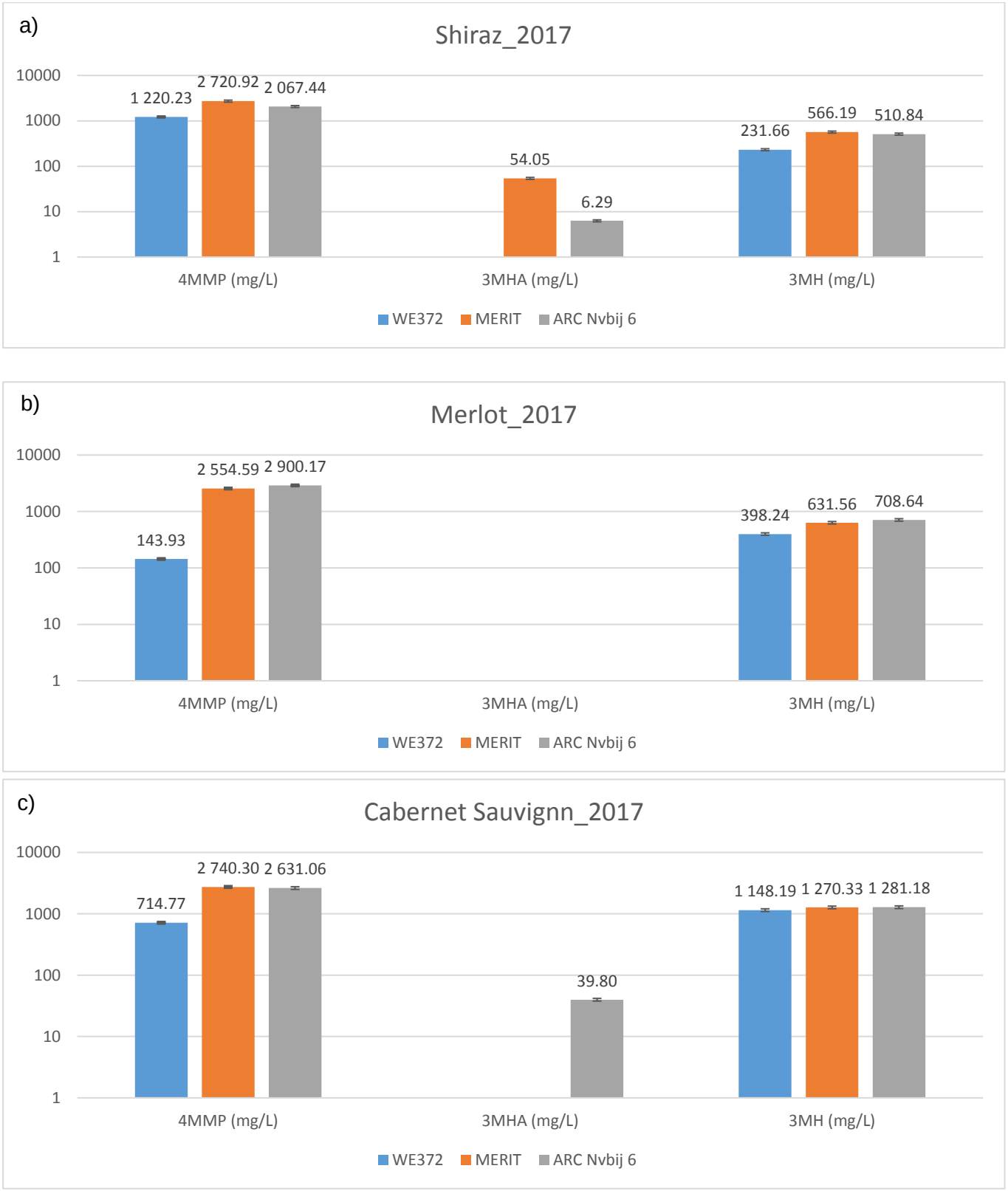

Figure 7. Concentrations of volatile thiols (4-mercapto-4-methylpentan-2-one, 4MMP; 3-mercaptohexan-1-ol, 3MH; and 3-mercaptohexyl acetate, 3MHA) measured using GC-FID for 2017 wines a) Shiraz, b) Merlot, and c) Cabernet Sauvignon.

3.4.7 Protein quantification and quality control

Alcoholic fermentation was initiated using three different *Saccharomyces cerevisiae* yeast strains *i.e.* natural experimental yeast ARC Nvbij 6 and the commercial yeasts MERIT and WE372. Tables 2 to 4 shows the chemical composition of the three grape cultivars *i.e* used, as a base must for the fermentations for both 2016 and 2017 vintages. A small amount of variation was observed within the same cultivar (Shiraz, Merlot, and Cabernet Sauvignon) between the two vintages (2016 and 2017) in terms of chemical composition. However, final wine chemical, sensory and metabolite profiles differed. As wine yeast proteins expressed during fermentation were previously reported to be effectors in the release of aroma compounds *e.g.* volatile thiols (Swiegers et al., 2007a; Moreno-Garcia 2015; Synos et al 2015), wine yeast protein expression at the start and the stationary phase of Shiraz grape must fermentation during the 2016 vintage was also investigated by deploying gel-based protocols. The objective was to investigate whether the yeast starter culture differentially expressed proteins at the beginning of fermentation compared to the end of fermentation.

The decision to focus on one cultivar was due to a study conducted by Moreno-García et al. (2015), which is one of the very few studies if not the only study thus far, that successfully managed to compare the proteome and exometabolome of a *S. cerevisiae* flor yeast strain in a biofilm and a non-biofilm. Furthermore, a wine also comprises plenty of tannins and polyphenols than are known to bind to yeast proteins (Mekoue Nguela et al., 2016), thus making proteome analysis difficult. This interaction with grape and/or wine derived compounds will, subsequently negatively influence protein yield during extractions. However, the relationship between the yeast proteome and exometabolome, and its influence on the organoleptic properties of wine remains unexplored and warrants further investigation.

3.4.1.1 Protein quantification using Bradford assays

A Bovine Serum Albumin (BSA) standard curve with absorbance as a function of protein concentration using the Bradford assay in conjunction with spectrophotometry (Abs 595 nm), showed a linear relationship of $R^2 = 0.9817$ (Fig. 8). Protein yield of fermenting *i.e.* commercial reference yeasts *i.e.* WE372 and MERIT and the experimental yeast *i.e.* ARC Nvbij 6 protein extracts obtained by extrapolating spectrophotometry data (Abs 595 nm) on a BSA standard curve following Bradford assay, ranged between 25 and 110 µg/µL (Fig. 9). Protein concentration was also determined by deploying another spectrophotometric approach *i.e.* NanoDrop™ UV-Vis spectrophotometer as a complementary approach, which also showed sufficient protein yield for all yeast strains (> 20 µg/µL) (data not shown).

Fermenting Shiraz grape must sampled at the start of fermentation showed that both commercial reference yeasts *i.e.* WE372 (109.7 µg/µL) and MERIT (59.7 µg/µL) had higher protein expression and secretion than the experimental yeast *i.e.* ARC Nvbij 6 (25.9 µg/µL) (Fig. 9). However, protein expression and secretion during the stationary phase of fermentation was similar for all strains, with ARC Nvbij 6 (76.3 µg/µL) even surpassing MERIT (69.8 µg/µL), whilst being marginally lower than WE372 (77.0 µg/µL). Nonetheless, protein concentrations in all the samples were sufficient to proceed with SDS-PAGE, as Coomassie Brilliant Blue G-250 has a detection threshold of 30 ng (0.03 µg) (Kang et al., 2002).

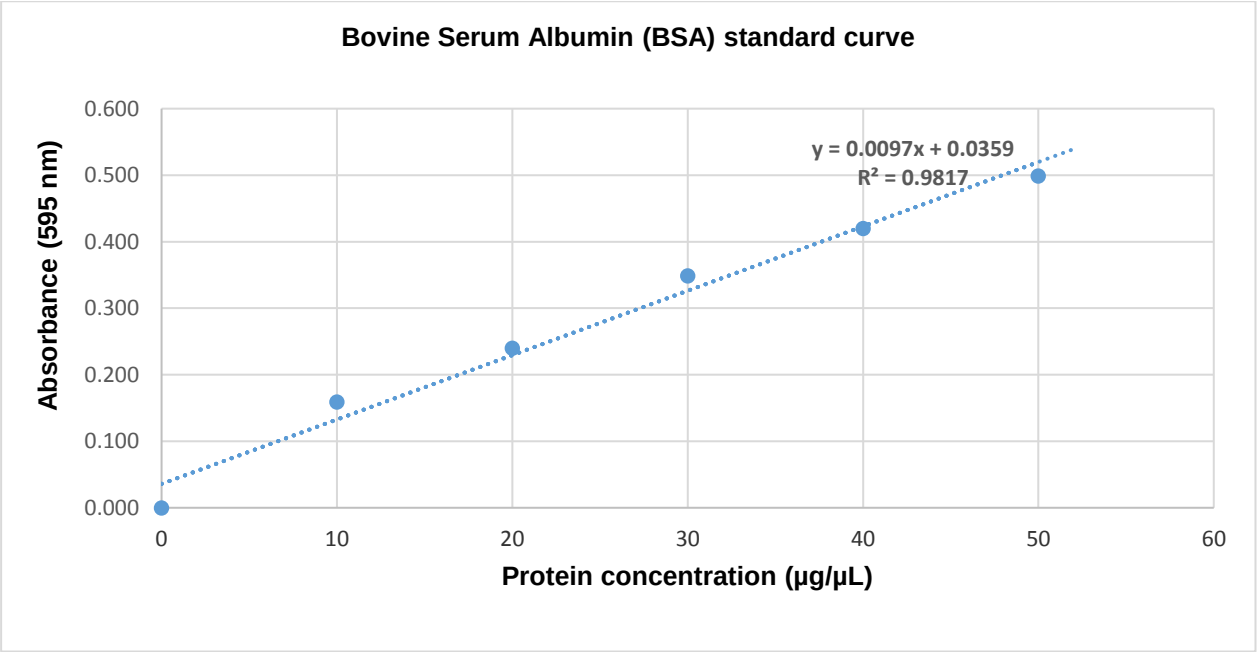

Figure 8. Bovine Serum Albumin (BSA) standard curve generating using Bradford assay spectrophotometric data measured at Abs 595 nm.

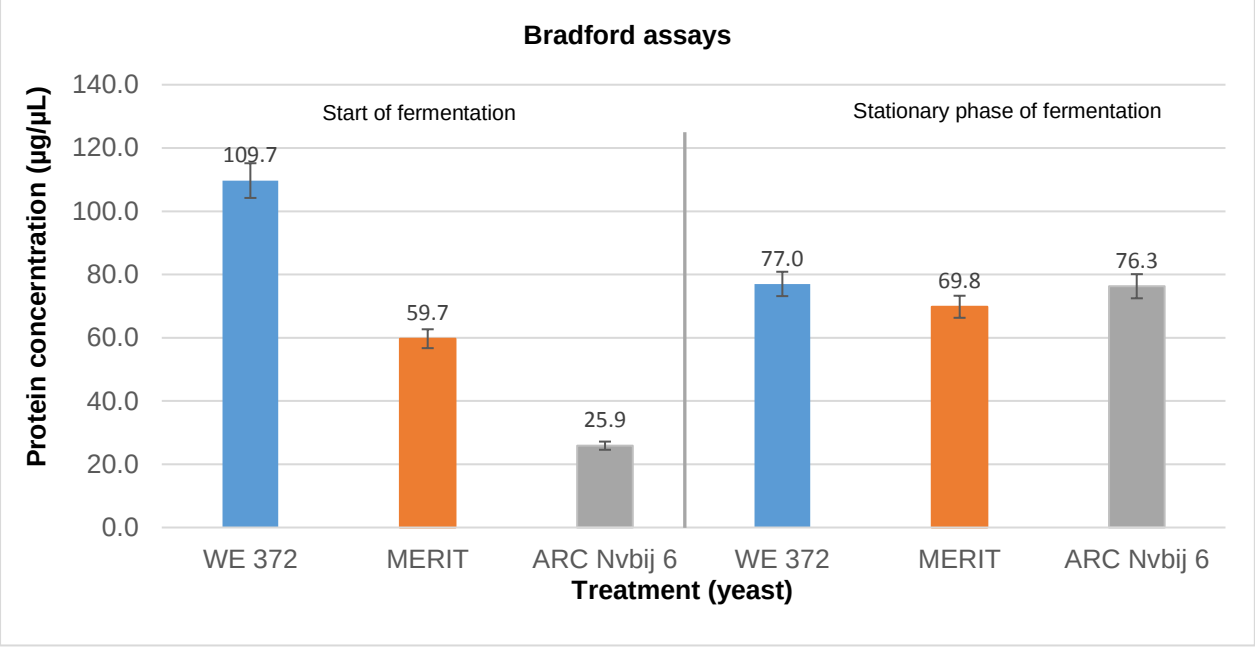

Figure 9. Protein concentration (µg/µL) of fermenting *i.e.* commercial reference yeasts *i.e.* WE372 and MERIT, and the experimental yeast *i.e.* ARC Nvbij 6 protein extracts obtained by extrapolating spectrophotometry data (Abs 595 nm) done in triplicate on a BSA standard curve following Bradford assay.

3.4.1.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Approximately 50 µg of protein originating from the respective yeast strains at the start and stationary phases of Shiraz grape must fermentation were loaded into wells prior to SDS-PAGE. Reason being, the intensity of the protein bands would then tentatively serve as an indication of down-regulated or overexpressed proteins within a given molecular weight (Hart et al et 2016). Ideally, proteins should have been separated using a second dimension *i.e.* isoelectric point (pI), which would have enabled us to differentiate proteins with equivalent molecular weights (viewed as one SDS-PAGE band) to pinpoint specific regulated proteins. Subsequently, differentially expressed proteins could have been identified by PDQuest™ software (Bio-Rad, Madrid, Spain). However, two-dimensional (2D) PAGE proved unsuccessful due to wine phenolic compounds interfering with isoelectric focusing. The SDS-PAGE showed marginal differences in protein banding profiles of the same yeast strain during the different phases as WE372 in lanes 2 and 5 (blue squares), MERIT in lanes 3 and 6 (yellow squares) and ARC Nvbij 6 in lanes 3 and 7 (red squares) showed different protein intensities in the > 25 kDa region. A similar observation was made for all strains with regard to a protein within the 38 to 50 kDa region too. Indications are that these proteins highlighted were differentially expressed due to changes in the grape must matrix as the fermentation progressed. It can, therefore tentatively be said that they were effectors in volatile aroma compound release as volatile compound levels in wines produced with different yeast strains differed. However, this notion will be confirmed in future by excising these proteins and subjecting it to in-gel digestion and identifying them by deploying peptide mass fingerprinting (PMF) in conjunction with matrix-assisted laser desorption ionization with time of flight mass spectrometry (MALDI -TOF MS) as previously reported by Ngara et al., 2012.

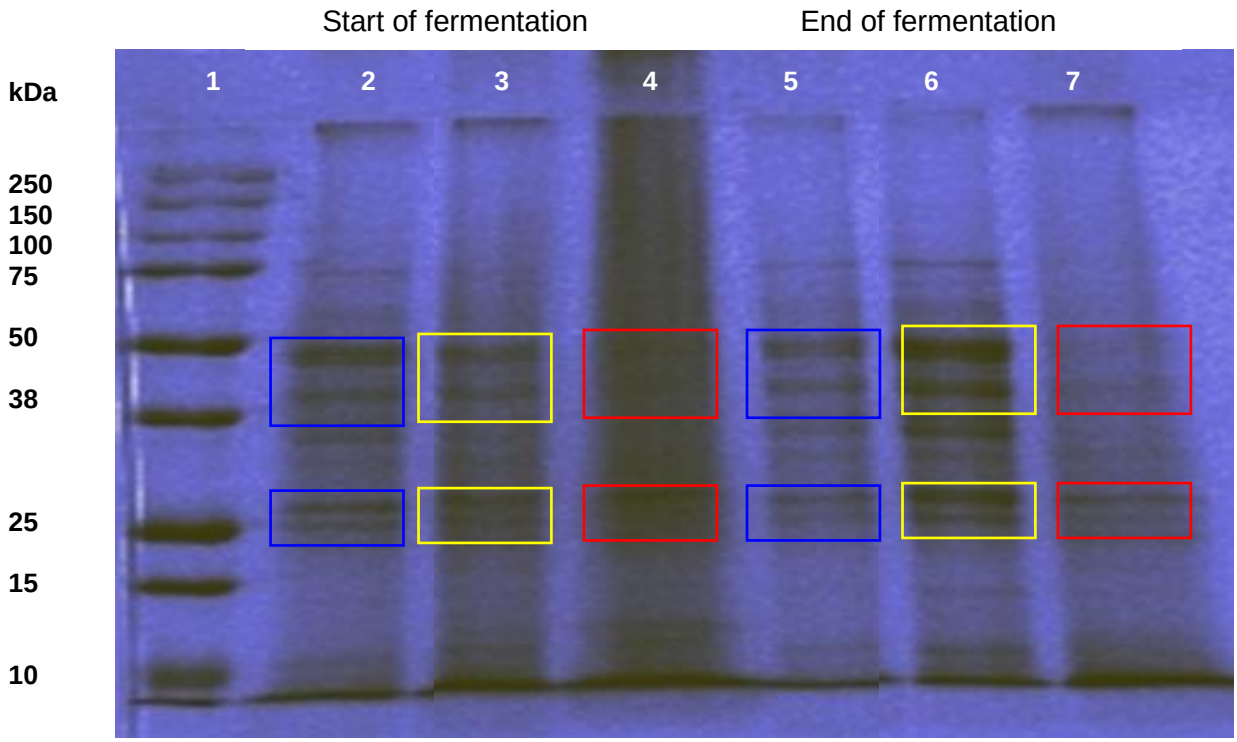

Figure 10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). of protein extracts originating from fermenting commercial *i.e.* WE372 and MERIT and experimental *i.e.* ARC Nvbij 6 yeasts sampled at the start and stationary phases of Shiraz grape must fermentation during the 2016 vintage. Lane 1: Protein ladder (Precision Plus Protein™, Bio-Rad, Madrid, Spain); Lanes 2 to 4: WE372, MERIT and ARC Nvbij 6, respectively (start of fermentation); lanes 5 to 7: WE372, MERIT and ARC Nvbij 6, respectively (stationary phase of fermentation).

3.5 CONCLUDING REMARKS

In conclusion, the experimental yeast strain *i.e.* ARC Nvbij 6 produced Shiraz, Merlot, Cabernet Sauvignon during the 2016 and 2017 vintages, equal and in some instances better than both commercial references *i.e.* WE372 and MERIT, respectively. It is noteworthy, that all wines produced with the experimental yeast *i.e.* ARC Nvbij 6 had a negative association with VA. Volatile acidity is undesirable as it gives wines and unpleasant off-odours, and can mask the sought-after varietal aromas and flavours (Swiegers et al., 2005; Hart et al., 2017a). All ARC Nvbij 6 produced wines also had the lowest acetic acid, which essentially is the main contributor to VA. This is a good observation as it shows that the experimental strain is a low VA producer irrespective of vintage and cultivar, therefore making it a good strain for typical varietal red wine production. This

notion was supported by red wine descriptive sensory evaluations, as the yeast produced Shiraz, Merlot and Cabernet Sauvignon wines with, amongst others, 'jammy', 'smokey', 'spicy', 'floral' and 'vegetative' aromas and flavours, all of which are associated with the above-mentioned cultivars. However, Merlot ('plum', 'berries' etc.) and Cabernet Sauvignon ('vegetative fresh', 'berry' etc.) wines produced with ARC Nvbij 6, had lower associations with sought-after aromas and flavours compared to the Shiraz wines. The yeast ARC Nvbij 6 also produced 2016 and 2017 Shiraz wines that complemented each other in terms of 'smoky' and 'spicy' aromas.

It is noteworthy that ARC Nvbij 6 also produced 2016 Shiraz wines with more 3MH (impart black currant aromas in red wines) (Blanchard et al., 2000) than both commercial references. This observation can tentatively be ascribed to the fact that the strain was isolated from Paarl regional Shiraz grapes. The yeast ARC Nvbij 6 was also shown to be a better '3MH to 3MHA converter', as both commercial references also failed to convert 3MH to 3MHA during one vintage in two cultivars. In terms of aroma compounds *i.e.* esters (associated with fruity nuances), both commercial references, by and large, produced Shiraz, Merlot and Cabernet Sauvignon wines with more ester concentrations than ARC Nvbij 6. Nonetheless, ARC Nvbij 6 consistently produced less of the undesirable compounds that are associated with wine off-odours, which can influence wine sensory quality negatively. These off-odours are also known to mask the effect of the sought-after compounds associated with 'fruity' aroma and flavour.

As wine yeast expressed and secreted proteins are instrumental in wine aroma compound release (Swiegers et al., 2007a), PMF and MALDI-TOF MS will be deployed to characterise yeast-derived proteins that were regulated to investigate how they are associated with aroma compounds. It is evident from SDS-PAGE that proteins within given molecular weights were differentially expressed. It is envisioned that proteomic analysis may be used to select promising wine yeast strains with sought-after traits in terms of wine quality (Trabalzini et al., 2003). The use of multiple omics approaches is also encouraged, as proteomics does effect metabolomics, which in turn determines wine chemical and sensory quality. Overall, ARC Nvbij 6 proved that it has a commercial role to play for the production varietal red wines, especially for Shiraz based chemical and sensory attributes.

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Chapter 4

General Discussion

CHAPTER 4: GENERAL DISCUSSION

4.1 SUMMARY OF FINDINGS

Wine yeast, particularly *Saccharomyces cerevisiae* has been the major yeast used to ferment *Vitis vinifera* grape must since 1866 when Louis Pasteur discovered that it was actually yeast that turned the grape must into wine or the pleasurable beverage it was formerly known as (Barnett, 2000; Jolly et al., 2014). Alcoholic fermentation conducted mostly by *S. cerevisiae* convert grape must into an alcoholic beverage containing numerous metabolites *e.g.* esters, higher alcohols, fatty acids etc. that give the wine its rich flavour and aroma (Fleet, 2003). Furthermore, it was believed that the fermenting yeast strain does not contribute to the varietal aroma of wine until the discovery of additional metabolites, namely volatile thiols. Varietal aroma of a wine is cultivar-specific, hence it was believed that it originates solely from the grape cultivar. The contention that thiols impact aroma started when Du Plessis and Augustyn (1981) proved that the guava aroma perceived in South African Sauvignon blanc wines strongly correlated with the occurrence of 4MMP. Hereafter volatile thiols attracted a lot of attention, especially in Sauvignon blanc wine. Volatile thiols was found to enhance the tropical fruit aroma and flavour of Sauvignon blanc which is a varietal character after being released by the fermenting yeast from its bound non-volatile cysteine precursors present in grape berries/must (Swiegers et al., 2007; Swiegers et al., 2009; Holt et al., 2011; Roncoroni et al., 2011). The importance of *S. cerevisiae* to wine technology and subsequently the wine industry is thus indisputable. However, the sensorial influence of thiols on red wine varietal aroma and flavour is still poorly studied, hence this aspect was also investigated in this study.

Surprisingly the workhorses, which are the proteins required to release, amongst others, volatile thiols, are not so well understood either. Proteins expressed during fermentation, their influence on the metabolites produced, and the subsequent influence on the aroma and flavour perceived in the wine remains unclear. Differential protein expression will be reflected in the phenotype (aroma and flavour) of the wines. Thus, this was one of the objectives of the current study. The current study was, therefore, initiated to investigate the influence of a naturally isolated

S. cerevisiae wine yeast strain *i.e.* ARC Nvbij6 on red wine varietal aroma and flavour by utilizing chemical, sensory, metabolomics and proteomic tools.

Our study demonstrated that the experimental yeast strain ARC Nvbij 6 consistently produced varietal Shiraz, Merlot, and Cabernet Sauvignon during the 2016 and 2017 vintages, equal and in some instances better than both commercial references (Chapter 3). Shiraz, Merlot and Cabernet Sauvignon wines produced with ARC Nvbij 6 also displayed sought-after aromas and flavours. However, WE372 produced better Merlot and Cabernet Sauvignon with a more positive association with varietal aromas and flavours than ARC Nvbij 6 and MERIT during the 2017 vintage.

Furthermore, basic chemical analyses data showed that ARC Nvbij 6 produced Shiraz, Merlot and Cabernet Sauvignon wines with the least volatile acidity (VA) compared to the wines produced by both commercial yeasts, namely WE372 and MERIT during both vintages.Our study also showed that VA levels between wines originating from different cultivars during both vintages produced with the same yeast strain *e.g*. ARC Nvbij 6 also differed. It is, therefore, clear that the yeast starter culture, vintage, as well as grape cultivar will influence final wine chemical and sensory attributes. Thus these factors have to be considered in the yeast development and selection process. Our study were in agreement with previous studies conducted by Du Plessis et al. (2017) and Hart et al. (2017), as different yeast strains produced wines with different organoleptic profiles. It is noteworthy that, ARC Nvbij 6 potentially has a commercial role to play, as it was was previously reported that low VA producers is an asset to the wine industry (Vilela et al., 2013). Reason being, unpleasant off-odours are known to mask sought-after varietal aromas and flavours.

In terms of aroma compounds *i.e.* esters (associated with fruity nuances), both commercial references mostly produced Shiraz, Merlot and Cabernet Sauvignon wines with higher ester concentrations than the ARC Nvbij 6 strain. Nonetheless, ARC Nvbij 6 consistently produced less of the undesirable compounds that are associated with wine off-odours, which can influence the wine sensory quality negatively. Our study also showed ARC Nvbij 6 to be a better '3MH to 3MHA converter', as both commercial references also failed to convert 3MH to 3MHA during one vintage in two cultivars. It is noteworthy that, ARC Nvbij 6 produced 2016 Merlot wines with noticeably

higher levels of 3MH than WE372, which suggests that 3MH might be associated with ‘floral’ and ‘violet’ aromas. As these volatile compounds have only recently been reported in red wines, this notion cannot be excluded. This is in agreement with Lapalus (2016) as they also found a link between certain volatiles and aromas perceived. In some instances thiol and ester concentrations were above their respective sensory thresholds, yet the sensory data did not reflect it. This phenonema was observed in all the strains This could be due to the masking effect of methoxypyrazines, responsible for the green aromas in Cabernet Sauvignon wines (Marais, 1994), as well as rotundone which is responsible for the peppery aroma commonly perceived in Shiraz wines (Wood et al., 2008). Further studies should be conducted on the antagonistic and enhancing interactions between metabolites responsible for green aromas and metabolites responsible for fruity aromas and flavours of red wines.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts originating from fermentating wines showed that different yeast strains were differentially expressed as protein banding profiles displayed marginal differences in terms of intensity (Chapter 3). It can, therefore, be tentatively said that differences in protein banding profiles given the fact that proteins are considred final effectors, were somehow linked to varying volatile aroma compound levels observed between different yeast strains. However, this notion will be confirmed in future by excising these proteins and subjecting it to in-gel digestion and identification by deploying PMF in conjunction with MALDI -TOF MS as previously reported by Ngara et al. (2012). Subsequently, associations can be made between specific yeast-derived proteins that were regulated and wine aroma compounds. Thus, proteomic tools may be used to select promising wine yeast strains with sought-after traits in terms of wine quality. The use of multiple omics approaches is also encouraged, as proteomics does affect metabolomics, which in turn determine wine chemical and sensory quality. Overall, the ARC Nvbij 6 strain proved that it has a commercial role to play in the production of varietal red wines, especially Shiraz, based on chemical and sensory attributes of all red wines included in this study**.**

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Appendix I

Descriptive sensory evaluation sheets

Sh

WINE SCORING SHEET

Judge: _____ Date: _____

Cultivar: **Shiraz** Wine number:

Judge the wine on the following line-scales:

VISUAL

Colour Unacceptable _____ Excellent

FLAVOUR (NOSE/TASTE INTENSITY)

	Undetectable	Prominent
Spicy	_____	
Smoky	_____	
Berry	_____	
Jammy	_____	
Vegetative	_____	

TASTE (INTENSITY)

Body (mouthfeel)	Thin _____	Full
Finish (long/short)	Short _____	Long

OVERALL QUALITY

Unacceptable _____ Excellent

Comments:.....

Descriptors:

Vegetative(Fresh)	-	Herbaceous, green cut-grass, green pepper, eucalyptus, mint.
(Cooked)	-	Green beans, asparagus, olives, artichoke.
(Dried)	-	Hay/straw, tea, tobacco.
Berry	-	Bramble, raspberry, strawberry, blackberry.
Spicy	-	Drop, aniseed, black pepper, cloves.
Other	-	Stone fruit (cherry, apricot, peach, apple, plum).

Processed fruit (strawberry jam, raisin, prunes, figs).

Figure 1. Shiraz wine descriptive sensory evaluation/scoring sheet indicatating aroma and flavour descriptors measured on a structured 10 cm line scale.

Mer

WINE SCORING SHEET

Judge: _____ Date: _____

Cultivar: **Merlot** Wine number:

Judge the wine on the following line-scales:

VISUAL

Unacceptable — Excellent

Colour _____

FLAVOUR (NOSE/TASTE INTENSITY)

Undetectable — Prominent

Spicy _____

Berry _____

Fruity _____

Floral (Violet) _____

TASTE (INTENSITY)

Thin — Full

Body (mouthfeel) _____

Short — Long

Finish (long/short) _____

OVERALL QUALITY

Unacceptable — Excellent

_____

Comments:.....

Descriptors:

Vegetative	(Fresh)	-	Herbaceous, green cut-grass, green pepper, eucalyptus, mint.
	(Cooked)	-	Green beans, asparagus, olives, artichoke.
	(Dried)	-	Hay/straw, tea, tobacco.
Berry		-	Bramble, raspberry, strawberry, blackcurrant.
Spicy		-	Drop, aniseed, black pepper, cloves.
Tree fruit		-	Plum, cherry, apricot, peach, apple.
Tropical fruit		-	Pineapple, musk-melon, banana, guava.
Dried fruit		-	Raisin, prune, peach, fig

Figure 2. Merlot wine descriptive sensory evaluation/scoring sheet indicatating aroma and flavour descriptors measured on a structured 10 cm line scale.

CS

WINE SCORING SHEET

Judge: _____ Date: _____

Cultivar: **Cabernet Sauvignon** Wine number:

Judge the wine on the following line-scales:

VISUAL		
	Unacceptable	Excellent
Colour	_____	

FLAVOUR (NOSE/TASTE INTENSITY)		
	Undetectable	Prominent
Spicy	_____	
Berry	_____	
Vegetative (fresh)	_____	
Vegetative (cooked)	_____	
Vegetative (dried)	_____	
Other	_____	

TASTE (INTENSITY)		
	Thin	Full
Body (mouthfeel)	_____	
	Short	Long
Finish (long/short)	_____	

OVERALL QUALITY		
	Unacceptable	Excellent
	_____	

Comments:.....

Descriptors:

Vegetative	(Fresh)	-	Herbaceous, green cut-grass, green pepper, eucalyptus, mint.
	(Cooked)	-	Green beans, asparagus, olives, artichoke.
	(Dried)	-	Hay/straw, tea, tobacco.
Berry		-	Bramble, raspberry, strawberry, blackcurrant.
Spicy		-	Drop, aniseed, black pepper, cloves.
Tree fruit		-	Plum, cherry, apricot, peach, apple.
Tropical fruit		-	Pineapple, musk-melon, banana, guava.
Dried fruit		-	Raisin, prune, peach, fig

Figure 3. Cabernet Sauvignon wine descriptive sensory evaluation/scoring sheet indicatating aroma and flavour descriptors measured on a structured 10 cm line scale.

Appendix II

Aroma compound analyses using GC-FID

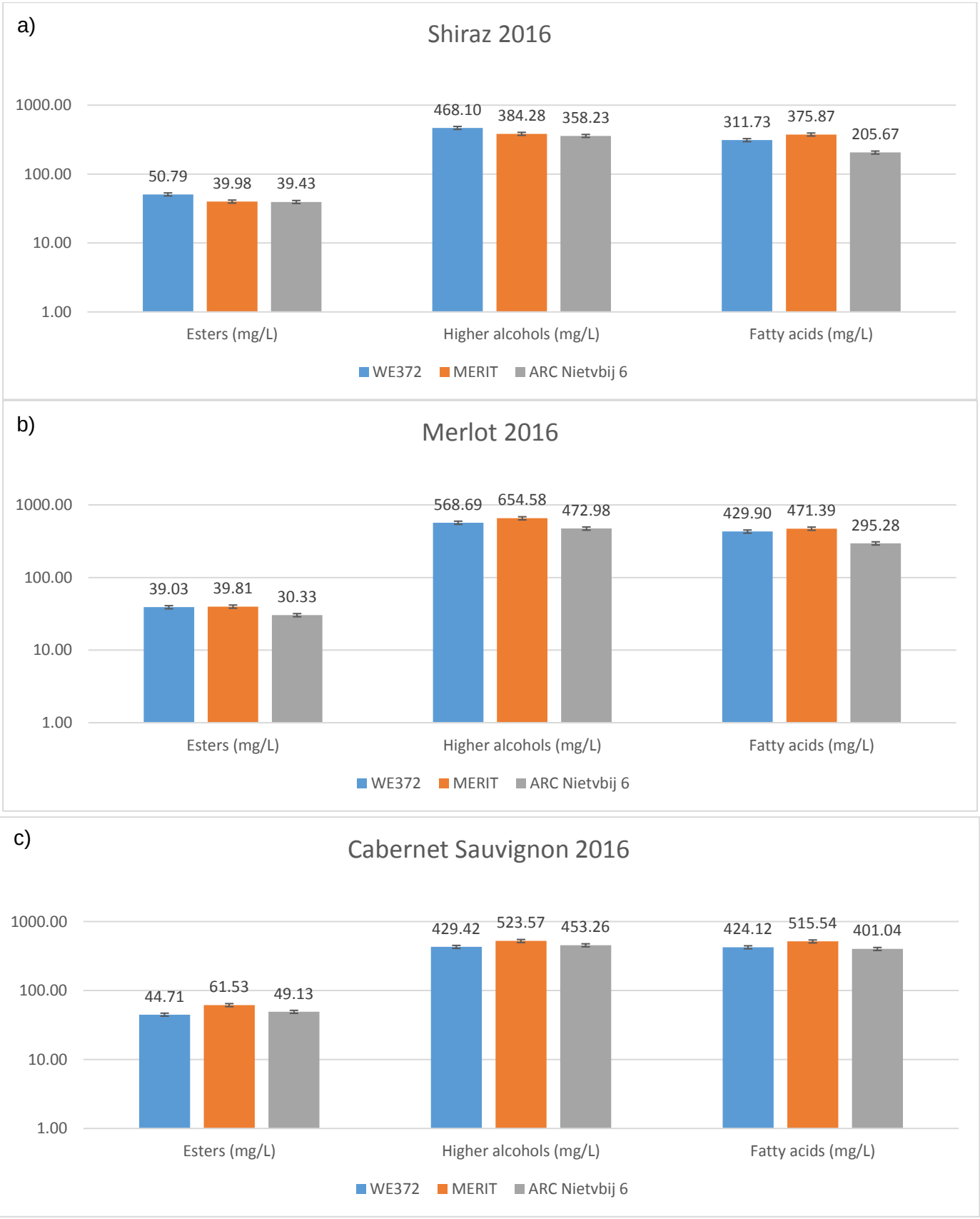

Figure 4. Total values of major volatile compounds (esters, higher alcohols, and fatty acids) measured using GC-FID for 2016 wines a) Shiraz, b) Merlot, and c) Cabernet Sauvignon.

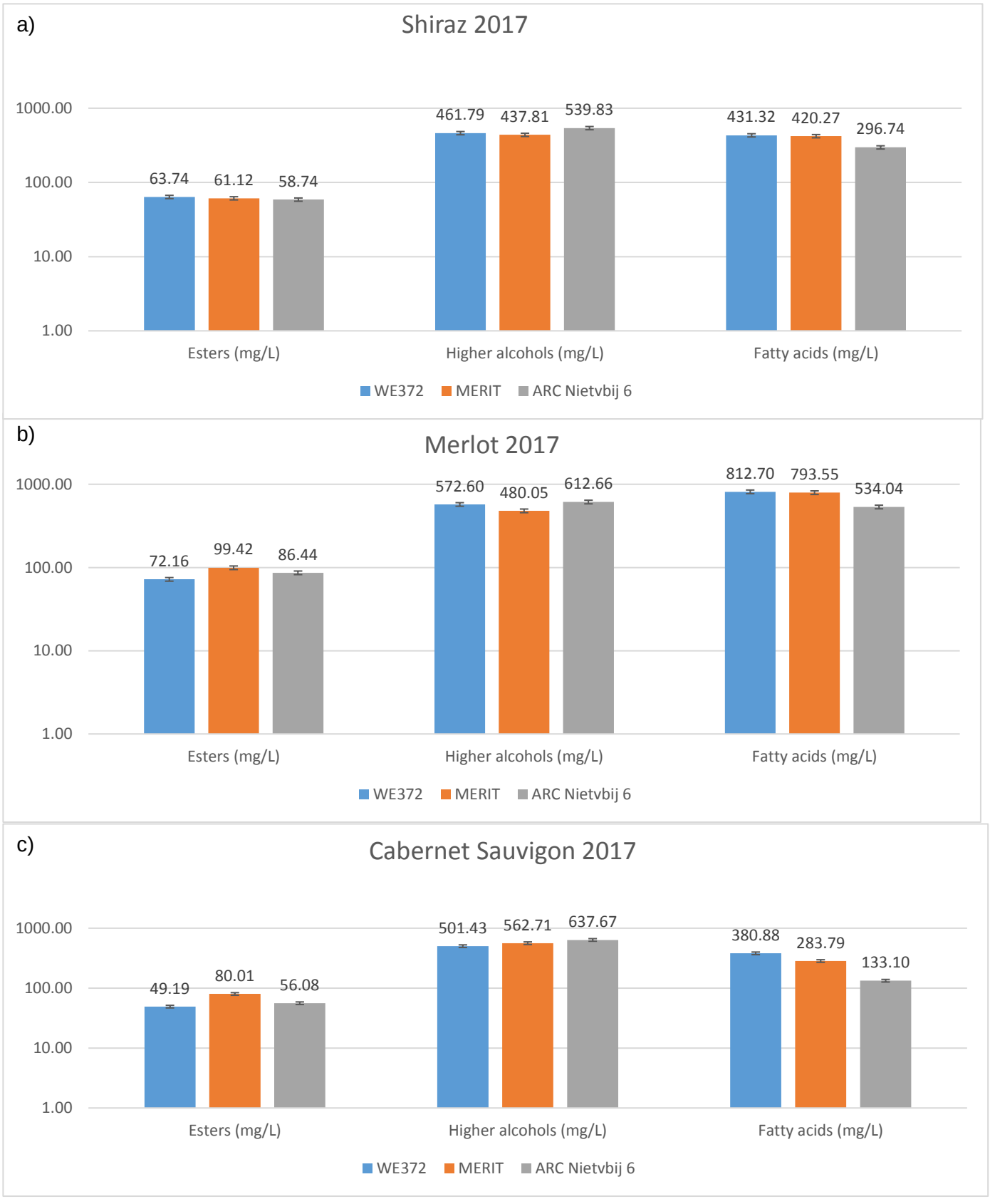

Figure 5. Total values of major volatile compounds (esters, higher alcohols, and fatty acids) measured using GC-FID for 2017 wines a) Shiraz, b) Merlot, and c) Cabernet Sauvignon.